

REMARKS

Claims 53, 58-72, 74, and 77-97 are pending in the application. No amendments to the claim are presented in this paper.

The previous rejections were withdrawn and a new ground of rejection presented, which is addressed below.

Claims 53, 58-72, 74, and 77-97 were rejected as allegedly not enabled. The Examiner contends that insufficient guidance is provided in the specification such that one of skill in the art could predict immunogenic fragments of SEQ ID NO:2 that would produce an anti-tumor immune response in a human patient. In particular, the Examiner argues that it is unpredictable that administration of immunogenic fragments of SEQ ID NO:2 would elicit a cellular immune response when administered to a subject that has a cancer that overexpresses PSCA. Applicants respectfully traverse.

As the Examiner knows, it is not the amount of experimentation or the complexity of the experimentation that determines enablement, but whether one of skill in the art could practice the invention without undue experimentation (*see, e.g., In re Wands*, 8 USPQ2d 1400 (Fed. Circ 1988)). As described in *Wands*, “a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should precede” (*see, Wands*, 8 USPQ2d at 1404, quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982)). Here, the experimentation required for practicing the invention may be time-consuming; but it is nonetheless routine. As explained below, the guidance in the specification coupled with the level of skill and knowledge in the art, permit a practitioner to identify and administer PSCA or PSCA fragments, that induce a cellular immune response with a reasonable expectation of success.

Applicants provide direction in the specification for practicing the claimed invention

The specification discloses how to make and use the invention to a practitioner in the art. First, the specification teaches the population to which the claimed PSCA protein, or

immunogenic fragment, is administered. For example, page 19, lines 7-10 of the application as filed discloses that PSCA is overexpressed by both androgen-dependent and androgen-independent prostate cancer cells (FIG. 9-11), prostate cancer metastases to bone (FIG. 20-24 and 26-32), bladder carcinomas (FIG. 6, 25 and 62), and pancreatic carcinomas (FIGS. 63 and 64). Further, page 51, lines 19-23 teaches that PSCA is a target for cancer immunotherapy, as it is expressed or overexpressed in a number of cancers.

Second, Applicants refer the practitioner to methodology known in the art as of the priority date of the application for additional guidance in identifying suitable polypeptides for administration. Page 60, lines 27-29, *e.g.*, cites references that explain how CTL epitopes that optimally bind to specified HLA alleles can be identified.

Third, Applicants have provided further direction for administering such protein or protein fragments. For example, passages at pages 60 -61 provide exemplary *in vivo* and *ex vivo* embodiments for administration of PSCA, or fragments thereof, to elicit T-cell-mediated immune responses. Accordingly, the specification provides a substantial amount of guidance to the practitioner.

The level of expertise and general knowledge in this art is advanced.

Applicants further note that this is an advanced field. The prior art teaches methods of identifying fragments that elicit cellular immune responses, both *in vitro* and *in vivo*. These techniques were well known as of the priority date of the invention. For example, WO 94/03205 and WO 94/020127, Appendices A and B, respectively, teach methods of identifying CTL epitopes that bind to HLA A2.1, HLA A1, HLA A3.2, HLA A11, and HLA A24.1. These publications provide descriptions of how to evaluate an amino acid sequence for the presence of a CTL epitope motif, how to evaluate HLA binding activity of peptides comprising the epitope, and how to determine immunogenicity, both *in vitro* and *in vivo*.

Moreover, the invention claimed in US Patent No. 6,037,135, which contains the teachings of WO94/03205, is directed to methods of making immunogenic peptides that comprise T cell epitopes (in this case, those that bind to HLA A1, HLA A3.2, HLA A11, and HLA 24.1) that induce cytotoxic T cell responses. For example, claims 1, 18, 35, and 52 set

forth steps for making immunogenic peptides that comprise motifs taught by the patentees, including steps for evaluating immunogenicity. Step (d) of these claims defines testing complexes for the ability to be recognized by cytotoxic T cell and to thereby induce a cytotoxic T cell response to the epitope. Dependent claims 13, 30, 47, and 64 set forth that this step can be determined *in vivo*. This issued U.S. Patent is presumed to be enabling. Accordingly, it provides compelling evidence that the amount of experimentation required for one of skill in the art to identify immunogenic epitopes to induce a cellular immune response *in vivo* for practicing the instant invention is not undue.

Immunogenic PSCA peptides have been identified

To further demonstrate that the claims are enabled, Applicants provide additional evidence that PSCA peptides that elicit an immunogenic response in humans can be identified. For example, Kiessling *et al.*, (*Int. J. Cancer*, 102:390-397, 2002, attached in Appendix C) defined immunogenic peptides of PSCA that are recognized by circulating CD8⁺ T cell from prostate cancer patient (*see*, the abstract, lines 8 and 9). The authors screened the PSCA sequence and identified potential epitopes based on HLA-A2.1 motifs. Corresponding peptides were analyzed for binding to HLA-A2.1. Peptides that bound with high and intermediate affinity were used to evaluate T cell reactivity in prostate cancer patients (page 392, column 2). Circulating CD8⁺ T cells reactive against two peptides in prostate cancer were identified. These peptides efficiently activated CD8⁺ effector T cells that were capable of destroying prostate cancer cells (page 390, last paragraph of column 2). The authors' conclusion is that their results emphasize the suitability of PSCA as a target molecule for prostate cancer immunotherapy (last paragraph, page 396).

Last, the Examiner argues that it would be unpredictable whether SEQ ID NO:2 or any immunogenic fragments of SEQ ID NO:2 would produce an anti-tumor immune response in a human patient. Applicants disagree. Cancer vaccines that elicit cellular immune responses to prostate cancer polypeptides are known in the art. For example, Eder *et al.*, (*Clin. Cancer Res.* 6:1632-1638, 2000, attached as Appendix D), teach a vaccinia virus vaccine that encodes prostate-specific antigen. The phase I trial showed a T cell-response to a 9-mer peptide derived

from PSA (*see, e.g.*, the last line of the abstract). Tjoa *et al.* (*CA Cancer J. Clin.*:49:117-128, 1999) attached as Appendix E) report that infusions of autologous dendritic cells loaded with two HLA-A2-specific prostate-specific membrane antigen peptides. This vaccine elicited cellular immune responses in patients (*e.g.*, the second full paragraph of page 120). Small *et al.* (*J. Clin. Oncology* 18:3894-3903, 2000, attached as appendix F) report that dendritic cells loaded with a prostatic acid phosphatase (PAP)-granulocyte-macrophage colony-stimulating factor fusion protein stimulated immune responses to PAP in patients, (*e.g.*, the "**Results**" section of the abstract). Further, cancer vaccines targeting different cancers, such as melanoma, have also elicited T-cell responses in patients (*see, e.g.*, Smith *et al.*, *J. Clin. Oncology* 21:1562-1273, 2003, Appendix G, which reports that patients receiving a synthetic HLA-A2 peptide to a melanoma antigen, gp100 mounted T-cell responses (abstract)). Thus, induction of cellular immune responses can in fact be achieved in cancer patients, including prostate cancer patients, by administering a polypeptide, or fragment such as a peptide that binds an HLA allele.

The Examiner is reminded that Applicants need not demonstrate clinical efficacy of a claimed invention ("it is improper for Office personnel to request evidence of safety in the treatment of humans or regarding the degree of effectiveness." MPEP § 2107.03.V). Applicants have taught, in view of the knowledge in the art, how to make and use immunogenic peptides as set forth in the claims. The art provides no rationale as to why PSCA polypeptide and/or polypeptides fragments would not be expected to also be immunogenic in a cancer patient that overexpresses PSCA. Indeed, the art supports the conclusion that the practitioner would be able to identify and administer polypeptides that elicit cellular immune response *in vivo* (explained above; *see, also, e.g.*, Kiessling *et al, supra*, page 390, the last paragraph of the first column bridging to the second column first full paragraph, which describes the advantages of PSCA for immunotherapy).

In summary, Applicants have provided direction in the specification and provided evidence that one of skill can, in fact, follow the guidance provided by the specification and using established techniques, reasonably expect to identify and administer PSCA/fragments to cancer patients and successfully elicit an immune response. Accordingly, Applicants have taught

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PATENT

how to make and use the invention to the extent required by the enablement standards.

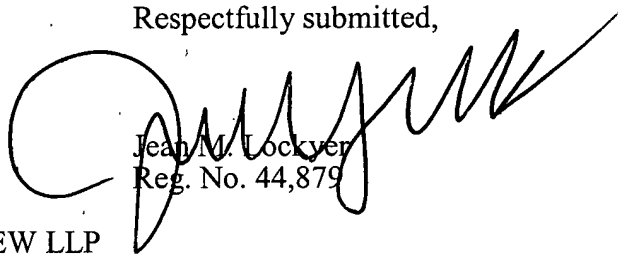
Applicants therefore respectfully request withdrawal of the invention.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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<p>(21) International Application Number: PCT/US94/02353</p> <p>(22) International Filing Date: 4 March 1994 (04.03.94)</p> <p>(30) Priority Data:</p> <table border="0"><tr><td>08/027,146</td><td>5 March 1993 (05.03.93)</td><td>US</td></tr><tr><td>08/073,205</td><td>4 June 1993 (04.06.93)</td><td>US</td></tr><tr><td>08/159,184</td><td>29 November 1993 (29.11.93)</td><td>US</td></tr></table> <p>(71) Applicant: CYTEL CORPORATION [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US).</p> <p>(72) Inventors: GREY, Howard, M.; 9066 La Jolla Shores Lane, La Jolla, CA 92037 (US). SETTE, Alessandro; 5551 Linda Rosa Avenue, La Jolla, CA 92037 (US). SIDNEY, John; 8541 D Villa La Jolla Drive, La Jolla, CA 92037 (US). KAST, W., Martin; Maria Rutgersweg 106, NL-2331 NX Leiden (NL).</p> <p>(74) Agents: BASTIAN, Kevin, L. et al.; Townsend and Townsend Khourie and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).</p>		08/027,146	5 March 1993 (05.03.93)	US	08/073,205	4 June 1993 (04.06.93)	US	08/159,184	29 November 1993 (29.11.93)	US	<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>
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<p>(54) Title: HLA-A2.1 BINDING PEPTIDES AND THEIR USES</p> <p>(57) Abstract</p> <p>The present invention provides the means and methods for selecting immunogenic peptides and the immunogenic peptide compositions capable of specifically binding glycoproteins encoded by HLA-A2.1 allele and inducing T cell activation in T cells restricted by the A2.1 allele. The peptides are useful to elicit an immune response against a desired antigen.</p>											

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HLA-A2.1 BINDING PEPTIDES AND THEIR USES

5 The present application is a continuation in part of
USSN 08/159,184, which is a continuation in part of USSN
08/073,205, which is a continuation in part of USSN
10 08/027,146, all of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

15 The present invention relates to compositions and
methods for preventing, treating or diagnosing a number of
pathological states such as viral diseases and cancers. In
particular, it provides novel peptides capable of binding
selected major histocompatibility complex (MHC) molecules and
inducing an immune response.

20 MHC molecules are classified as either Class I or
Class II molecules. Class II MHC molecules are expressed
primarily on cells involved in initiating and sustaining
immune responses, such as T lymphocytes, B lymphocytes,
macrophages, etc. Class II MHC molecules are recognized by
helper T lymphocytes and induce proliferation of helper T
lymphocytes and amplification of the immune response to the
25 particular immunogenic peptide that is displayed. Class I MHC
molecules are expressed on almost all nucleated cells and are
recognized by cytotoxic T lymphocytes (CTLs), which then
destroy the antigen-bearing cells. CTLs are particularly
important in tumor rejection and in fighting viral infections.

30 The CTL recognizes the antigen in the form of a
peptide fragment bound to the MHC class I molecules rather
than the intact foreign antigen itself. The antigen must
normally be endogenously synthesized by the cell, and a
portion of the protein antigen is degraded into small peptide
35 fragments in the cytoplasm. Some of these small peptides
translocate into a pre-Golgi compartment and interact with
class I heavy chains to facilitate proper folding and
association with the subunit $\beta 2$ microglobulin. The

peptide-MHC class I complex is then routed to the cell surface for expression and potential recognition by specific CTLs.

Investigations of the crystal structure of the human MHC class I molecule, HLA-A2.1, indicate that a peptide
5 binding groove is created by the folding of the $\alpha 1$ and $\alpha 2$ domains of the class I heavy chain (Bjorkman et al., Nature 329:506 (1987)). In these investigations, however, the identity of peptides bound to the groove was not determined.

Buus et al., Science 242:1065 (1988) first described
10 a method for acid elution of bound peptides from MHC.

Subsequently, Rammensee and his coworkers (Falk et al., Nature 351:290 (1991) have developed an approach to characterize naturally processed peptides bound to class I molecules.

Other investigators have successfully achieved direct amino
15 acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from class I molecules of the B type (Jardetzky, et al., Nature 353:326 (1991) and of the A2.1 type by mass spectrometry (Hunt, et al., Science 225:1261 (1992)). A review
20 of the characterization of naturally processed peptides in MHC Class I has been presented by Rötzschke and Falk (Rötzschke and Falk, Immunol. Today 12:447 (1991)).

Sette et al., Proc. Natl. Acad. Sci. USA 86:3296 (1989) showed that MHC allele specific motifs could be used to
25 predict MHC binding capacity. Schaeffer et al., Proc. Natl. Acad. Sci. USA 86:4649 (1989) showed that MHC binding was related to immunogenicity. Several authors (De Bruijn et al., Eur. J. Immunol., 21:2963-2970 (1991); Pamer et al., 991
30 Nature 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Class I motifs specific for a number of human alleles of a given class I isotype have yet to be described. It is desirable that the combined frequencies of these different
35 alleles should be high enough to cover a large fraction or perhaps the majority of the human outbred population.

Despite the developments in the art, the prior art has yet to provide a useful human peptide-based vaccine or

therapeutic agent based on this work. The present invention provides these and other advantages.

SUMMARY OF THE INVENTION

5 The present invention provides compositions comprising immunogenic peptides having binding motifs for HLA-A2.1 molecules. The immunogenic peptides, which bind to the appropriate MHC allele, are preferably 9 to 10 residues in length and comprise conserved residues at certain positions 10 such as positions 2 and 9. Moreover, the peptides do not comprise negative binding residues as defined herein at other positions such as positions 1, 3, 6 and/or 7 in the case of peptides 9 amino acids in length and positions 1, 3, 4, 5, 7, 8 and/or 9 in the case of peptides 10 amino acids in length. 15 The present invention defines positions within a motif enabling the selection of peptides which will bind efficiently to HLA A2.1.

 Epitopes on a number of immunogenic target proteins can be identified using the peptides of the invention. 20 Examples of suitable antigens include prostate cancer specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. The peptides are thus useful in pharmaceutical 25 compositions for both in vivo and ex vivo therapeutic and diagnostic applications.

Definitions

 The term "peptide" is used interchangeably with 30 "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The oligopeptides of the invention are less than about 15 residues 35 in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues.

 An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will

bind an MHC molecule and induce a CTL response. Immunogenic peptides of the invention are capable of binding to an appropriate HLA-A2.1 molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

Immunogenic peptides are conveniently identified using the algorithms of the invention. The algorithms are mathematical procedures that produce a score which enables the selection of immunogenic peptides. Typically one uses the algorithmic score with a "binding threshold" to enable selection of peptides that have a high probability of binding at a certain affinity and will in turn be immunogenic. The algorithm is based upon either the effects on MHC binding of a particular amino acid at a particular position of a peptide or the effects on binding of a particular substitution in a motif containing peptide.

A "conserved residue" is an amino acid which occurs in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide. Typically a conserved residue is one where the MHC structure may provide a contact point with the immunogenic peptide. One to three, preferably two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself. Typically, an immunogenic peptide will comprise up to three conserved residues, more usually two conserved residues.

As used herein, "negative binding residues" are amino acids which if present at certain positions (for example, positions 1, 3 and/or 7 of a 9-mer) will result in a peptide being a nonbinder or poor binder and in turn fail to be immunogenic i.e. induce a CTL response.

The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 8 to about 11 amino acids, which is recognized by a particular MHC allele. The peptide motifs are typically different for each human MHC

allele and differ in the pattern of the highly conserved residues and negative residues.

The binding motif for an allele can be defined with increasing degrees of precision. In one case, all of the conserved residues are present in the correct positions in a peptide and there are no negative residues in positions 1,3 and/or 7.

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their in situ environment, e.g., MHC I molecules on antigen presenting cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification scheme.

Fig. 2 shows a scattergram of the log of relative binding plotted against the "Grouped Ratio" algorithm for 9 mer peptides.

Fig. 3 shows a scattergram of the log of relative binding plotted against the average "Log of Binding" algorithm score for 9 mer peptides.

Figs. 4 and 5 show scattergrams of a set of 10-mer peptides containing preferred residues in positions 2 and 10 as scored by the "Grouped Ratio" and "Log of Binding" algorithms.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes, in particular, peptide motifs recognized by HLA-A2.1 alleles. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral diseases, cancers or autoimmune diseases, for which the amino acid sequence of the potential antigen or autoantigen targets is known.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

The peptides of the invention may also be employed to relieve the symptoms of, treat or prevent the occurrence or reoccurrence of autoimmune diseases. Such diseases include, for example, multiple sclerosis (MS), rheumatoid arthritis (RA), Sjogren syndrome, scleroderma, polymyositis, dermatomyositis, systemic lupus erythematosus, juvenile rheumatoid arthritis, ankylosing spondylitis, myasthenia gravis (MG), bullous pemphigoid (antibodies to basement membrane at dermal-epidermal junction), pemphigus (antibodies to mucopolysaccharide protein complex or intracellular cement substance), glomerulonephritis (antibodies to glomerular basement membrane), Goodpasture's syndrome, autoimmune hemolytic anemia (antibodies to erythrocytes), Hashimoto's disease (antibodies to thyroid), pernicious anemia (antibodies to intrinsic factor), idiopathic thrombocytopenic purpura (antibodies to platelets), Grave's disease, and Addison's disease (antibodies to thyroglobulin), and the like.

The autoantigens associated with a number of these diseases have been identified. For example, in experimentally induced autoimmune diseases, antigens involved in pathogenesis have been characterized: in arthritis in rat and mouse,

native type-II collagen is identified in collagen-induced arthritis, and mycobacterial heat shock protein in adjuvant arthritis; thyroglobulin has been identified in experimental allergic thyroiditis (EAT) in mouse; acetyl choline receptor (AChR) in experimental allergic myasthenia gravis (EAMG); and myelin basic protein (MBP) and proteolipid protein (PLP) in experimental allergic encephalomyelitis (EAE) in mouse and rat. In addition, target antigens have been identified in humans: type-II collagen in human rheumatoid arthritis; and acetyl choline receptor in myasthenia gravis.

Peptides comprising the epitopes from these antigens are synthesized and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorometry, peptide-dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or in vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles. The peptide binding motifs of the invention are relatively specific for each allelic subtype.

For peptide-based vaccines, the peptides of the present invention preferably comprise a motif recognized by an MHC I molecule having a wide distribution in the human population. Since the MHC alleles occur at different frequencies within different ethnic groups and races, the choice of target MHC allele may depend upon the target population. Table 1 shows the frequency of various alleles at

the HLA-A locus products among different races. For instance, the majority of the Caucasoid population can be covered by peptides which bind to four HLA-A allele subtypes, specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the
5 majority of the Asian population is encompassed with the addition of peptides binding to a fifth allele HLA-A11.2.

these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class I molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a combination thereof.

Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]).

Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The

capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecule binding assay as described in Example 4, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S (Melief, et al., Eur. J. Immunol. 21:2963 (1991)).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, Antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., Nature, 319:675 (1986); Ljunggren, et al., Eur. J. Immunol. 21:2963-2970 (1991)), and the human somatic T cell hybrid, T-2 (Cerundolo, et al., Nature 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce in vitro primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line (see Schneider J. Embryol. Exp. Morphol. 27:353-365 [1927]).

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100 μ M of

these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

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Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]). Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The

capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecule binding assay as described in Example 4, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S (Melief, et al., Eur. J. Immunol. 21:2963 (1991)).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, Antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., Nature, 319:675 (1986); Ljunggren, et al., Eur. J. Immunol. 21:2963-2970 (1991)), and the human somatic T cell hybrid, T-2 (Cerundolo, et al., Nature 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce in vitro primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATCC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line (see Schneider J. Embryol. Exp. Morphol. 27:353-365 [1927]).

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100 μ M of

peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded antigen-presenting cells are then incubated with the responder cell populations in vitro for 7 to 10 days under optimized culture conditions.

5 Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide
10 sequence was derived.

Specificity and MHC restriction of the CTL is determined by testing against different peptide target cells expressing appropriate or inappropriate human MHC class I. The peptides that test positive in the MHC binding assays and
15 give rise to specific CTL responses are referred to herein as immunogenic peptides.

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or from natural sources such as whole viruses or tumors. Although the
20 peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

The polypeptides or peptides can be a variety of
25 lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the
30 polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of
35 about 8 to about 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase Peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L- α -amino acids, or their D-isomers, but may include non-natural amino acids as well, such as β - γ - δ -amino acids, as well as many derivatives of L- α -amino acids.

Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 2 when it is desired to finely modulate the characteristics of the peptide.

TABLE 2

<u>Original Residue</u>	<u>Exemplary Substitution</u>
Ala	Ser
Arg	Lys, His
Asn	Gln
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Lys; Arg
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; His
Met	Leu; Ile
Phe	Tyr; Trp
Ser	Thr
Thr	Ser
Trp	Tyr; Phe
Tyr	Trp; Phe
Val	Ile; Leu

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al.,

Eur. J. Drug Metab. Pharmacokin. 11:291-302 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB,
5 non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous
10 trichloroacetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

15 The peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide desired attributes other than improved serum half life. For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which
20 contains at least one epitope that is capable of inducing a T helper cell response.

In some embodiments, the T helper peptide is one that is recognized by T helper cells in the majority of the population. This can be accomplished by selecting amino acid
25 sequences that bind to many, most, or all of the MHC class II molecules. These are known as "loosely MHC-restricted" T helper sequences. Examples of amino acid sequences that are loosely MHC-restricted include sequences from antigens such as Tetanus toxin at positions 830-843 (QYIKANSKFIGITE),
30 *Plasmodium falciparum* CS protein at positions 378-398 (DIEKKIAKMEKASSVFNVVNS), and Streptococcus 18kD protein at positions 1-16 (YGAVDSILGGVATYGAA).

Alternatively, it is possible to prepare synthetic peptides capable of stimulating T helper lymphocytes, in a
35 loosely MHC-restricted fashion, using amino acid sequences not found in nature. These synthetic compounds called Pan-DR-binding epitope (PADRE) are designed on the basis of

their binding activity to most, HLA-DR (human MHC class II) molecules (see, copending application USSN 08/121,101).

Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not comprise the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated. Exemplary T helper peptides include tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes CTL. Lipids have been identified as agents capable of priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, E. coli lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine (P_3 CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., Nature 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P_3 CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with P_3 CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support, or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal- NH_2 acylation, e.g., by alkanoyl (C_1 - C_{20}) or thioglycolyl acetylation, terminal-carboxyl amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), supra.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of

diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

5 For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides
10 separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or
15 complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general
20 state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 μ g to about 5000 μ g of peptide for a 70 kg patient, followed by boosting dosages of from
25 about 1.0 μ g to about 1000 μ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may
30 generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating
35 physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral infection or the detection or surgical

removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by
5 boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic
10 infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for
15 administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide
20 in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0 μg to about 5000 μg , preferably about 5 μg to 1000 μg for a 70 kg patient per dose. Immunizing doses
25 followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory
30 tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions
35 are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides

dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be
5 sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain
10 pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium
15 chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be
20 selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a
25 particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In
30 these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic
35 compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions.

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an

aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P₃CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or

otherwise at risk of viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 μg to about 5000 μg per 70 kilogram patient, more commonly from about 10 μg to about 500 μg mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL ex vivo, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the

patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand
5 into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell).

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to
10 determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be
15 used to predict which individuals will be at substantial risk for developing chronic infection.

The following examples are offered by way of illustration, not by way of limitation.

Example 1Class I antigen isolation

A flow diagram of an HLA-A antigen purification scheme is presented in Figure 1. Briefly, the cells bearing the appropriate allele were grown in large batches (6-8 liters yielding $\sim 5 \times 10^9$ cells), harvested by centrifugation and washed. All cell lines were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics. For large-scale cultures, cells were grown in roller bottle culture in RPMI 1640 with 10% FBS or with 10% horse serum and antibiotics. Cells were harvested by centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259 rotor and washed three times with phosphate-buffered saline (PBS) (0.01 M PO_4 , 0.154 M NaCl, pH 7.2).

Cells were pelleted and stored at -70°C or treated with detergent lysing solution to prepare detergent lysates. Cell lysates were prepared by the addition of stock detergent solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci. Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0] to the cell pellets (previously counted) at a ratio of 50-100 $\times 10^6$ cells per ml detergent solution. A cocktail of protease inhibitors was added to the premeasured volume of stock detergent solution immediately prior to the addition to the cell pellet. Addition of the protease inhibitor cocktail produced final concentrations of the following: phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5 $\mu\text{g/ml}$; leupeptin, 10 $\mu\text{g/ml}$; pepstatin, 10 $\mu\text{g/ml}$; iodoacetamide, 100 μM ; and EDTA, 3 ng/ml. Cell lysis was allowed to proceed at 4°C for 1 hour with periodic mixing. Routinely 5-10 $\times 10^9$ cells were lysed in 50-100 ml of detergent solution. The lysate was clarified by centrifugation at 15,000 $\times g$ for 30 minutes at 4°C and subsequent passage of the supernatant fraction through a 0.2 μ filter unit (Nalgene).

The HLA-A antigen purification was achieved using affinity columns prepared with mAb-conjugated Sepharose beads. For antibody production, cells were grown in RPMI with 10% FBS in large tissue culture flasks (Corning 25160-225).

Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein-A-Sepharose (Sigma). Briefly, saturated ammonium sulfate was added slowly with stirring to the tissue culture supernatant to 45% (volume to volume) overnight at 4°C to precipitate the immunoglobulins. The precipitated proteins were harvested by centrifugation at 10,000 x g for 30 minutes. The precipitate was then dissolved in a minimum volume of PBS and transferred to dialysis tubing (Spectro/Por 2, Mol. wt. cutoff 12,000-14,000, Spectrum Medical Ind.). Dialysis was against PBS (≥ 20 times the protein solution volume) with 4-6 changes of dialysis buffer over a 24-48 hour period at 4°C. The dialyzed protein solution was clarified by centrifugation (10,000 x g for 30 minutes) and the pH of the solution adjusted to pH 8.0 with 1N NaOH. Protein-A-Sepharose (Sigma) was hydrated according to the manufacturer's instructions, and a protein-A-Sepharose column was prepared. A column of 10 ml bed volume typically binds 50-100 mg of mouse IgG.

The protein sample was loaded onto the protein-A-Sepharose column using a peristaltic pump for large loading volumes or by gravity for smaller volumes (<100 ml). The column was washed with several volumes of PBS, and the eluate was monitored at A280 in a spectrophotometer until base line was reached. The bound antibody was eluted using 0.1 M citric acid at suitable pH (adjusted to the appropriate pH with 1N NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base was used to neutralize the eluate. Fractions containing the antibody (monitored by A280) were pooled, dialyzed against PBS and further concentrated using an Amicon Stirred Cell system (Amicon Model 8050 with YM30 membrane). The anti-A2 mAb, BB7.2, was useful for affinity purification.

The HLA-A antigen was purified using affinity columns prepared with mAb-conjugated Sepharose beads. The affinity columns were prepared by incubating protein-A-Sepharose beads (Sigma) with affinity-purified mAb as described above. Five to 10 mg of mAb per ml of bead is the preferred ratio. The

mAb bound beads were washed with borate buffer (borate buffer: 100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the washes show A280 at based line. Dimethyl pimelimidate (20 mM) in 200 mM triethanolamine was added to covalently crosslink the bound mAb to the protein-A-Sepharose (Schneider et al., J. Biol. Chem. 257:10766 (1982)). After incubation for 45 minutes at room temperature on a rotator, the excess crosslinking reagent was removed by washing the beads twice with 10-20 ml of 20 mM ethanolamine, pH 8.2. Between each one the slurry was placed on a rotator for 5 minutes at room temperature. The beads were washed with borate buffer and with PBS plus 0.02% sodium azide.

The cell lysate ($5-10 \times 10^9$ cell equivalents) was then slowly passed over a 5-10 ml affinity column (flow rate of 0.1-0.25 ml per minute) to allow the binding of the antigen to the immobilized antibody. After the lysate was allowed to pass through the column, the column was washed sequentially with 20 column volumes of detergent stock solution plus 0.1% sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0. The HLA-A antigen bound to the mAb was eluted with a basic buffer solution (50 mM diethylamine in water). As an alternative, acid solutions such as 0.15-0.25 M acetic acid were also used to elute the bound antigen. An aliquot of the eluate (1/50) was removed for protein quantification using either a colorimetric assay (BCA assay, Pierce) or by SDS-PAGE, or both. SDS-PAGE analysis was performed as described by Laemmli (Laemmli, U.K., Nature 227:680 (1970)) using known amounts of bovine serum albumin (Sigma) as a protein standard. Allele specific antibodies were used to purify the specific MHC molecule. In the case of HLA-A2, the mAb BB7.2 was used.

Example 2

Isolation and sequencing of naturally processed peptides

For the HLA-A preparations derived from the base (50 mM diethylamine) elution protocol, the eluate was immediately neutralized with 1 N acetic acid to pH 7.0-7.5. The neutralized eluate was concentrated to a volume of 1-2 ml in

an Amicon stirred cell [Model 8050, with YM3 membranes (Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was added to the concentrator to remove the non-volatile salts, and the sample was concentrated to approximately 1 ml. A
5 small sample (1/50) was removed for protein quantitation as described above. The remainder was recovered into a 15 ml polypropylene conical centrifuge tube (Falcon, 2097) (Becton Dickinson). Glacial acetic acid was added to obtain a final concentration of 10% acetic acid. The acidified sample was
10 placed in a boiling water bath for 5 minutes to allow for the dissociation of the bound peptides. The sample was cooled on ice, returned to the concentrator and the filtrate was collected. Additional aliquots of 10% acetic acid (1-2 ml) were added to the concentrator, and this filtrate was pooled
15 with the original filtrate. Finally, 1-2 ml of distilled water was added to the concentrator, and this filtrate was pooled as well.

The retentate contains the bulk of the HLA-A heavy chain and β_2 -microglobulin, while the filtrate contains the
20 naturally processed bound peptides and other components with molecular weights less than about 3000. The pooled filtrate material was lyophilized in order to concentrate the peptide fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography)
25 separation of the peptide fractions, the lyophilized sample was dissolved in 50 μ l of distilled water, or into 0.1% trifluoroacetic acid (TFA) (Applied Biosystems) in water and injected to a C18 reverse-phase narrow bore column (Beckman C18 Ultrasphere, 10 x 250 mm), using a gradient system
30 described by Stone and Williams (Stone, K.L. and Williams K.R., in, Macromolecular Sequencing and Synthesis; Selected Methods and Applications, A.R. Liss, New York, 1988, pp. 7-24. Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The
35 flow rate was 0.250 ml/minute with the following gradient: 0-60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-98% B. The Gilson narrow bore HPLC configuration is

particularly useful for this purpose, although other configurations work equally well.

A large number of peaks were detected by absorbance at 214 nm, many of which appear to be of low abundance. Whether
5 a given peak represents a single peptide or a peptide mixture was not determined. Pooled fractions were then sequenced to determine motifs specific for each allele as described below.

Pooled peptide fractions, prepared as described above were analyzed by automated Edman sequencing using the Applied
10 Biosystems Model 477A automated sequencer. The sequencing method is based on the technique developed by Pehr Edman in the 1950s for the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids.

15 The protein or peptide to be sequenced was held by a 12-mm diameter porous glass fiber filter disk in a heated, argon-purged reaction chamber. The filter was generally pre-treated with BioBrene PlusTM and then cycled through one or more repetitions of the Edman reaction to reduce contaminants
20 and improve the efficiency of subsequent sample sequencing. Following the pre-treatment of the filter, a solution of the sample protein or peptide (10 pmol-5 nmol range) was loaded onto the glass filter and dried. Thus, the sample was left embedded in the film of the pre-treated disk. Covalent
25 attachment of the sample to the filter was usually not necessary because the Edman chemistry utilized relatively apolar solvents, in which proteins and peptides are poorly soluble.

Briefly, the Edman degradation reaction has three
30 steps: coupling, cleavage, and conversion. In coupling step, phenylisothiocyanate (PITC) is added. The PITC reacts quantitatively with the free amino-terminal amino acid of the protein to form the phenylthiocarbamyl-protein in a basic environment. After a period of time for the coupling step,
35 the excess chemicals are extracted and the highly volatile organic acid, trifluoroacetic acid, TFA, is used to cleave the PITC-coupled amino acid residue from the amino terminus of the protein yielding the anilinothiazolinone (ATZ) derivative of

the amino acid. The remaining protein/peptide is left with a new amino terminus and is ready for the next Edman cycle. The ATZ amino acid is extracted and transferred to a conversion flask, where upon addition of 25% TFA in water, the ATZ amino acid is converted to the more stable phenylthiohydantoin (PTH) amino acid that can be identified and quantified following automatic injection into the Model 120 PTH Analyzer which uses a microbore C-18 reverse-phase HPLC column for the analysis.

In the present procedures, peptide mixtures were loaded onto the glass filters. Thus, a single amino acid sequence usually does not result. Rather, mixtures of amino acids in different yield are found. When the particular residue is conserved among the peptides being sequenced, increased yield for that amino acid is observed.

Example 3

Definition of an A2.1 specific motif

In one case, pooled peptide fractions prepared as described in Example 2 above were obtained from HLA-A2.1 homozygous cell lines, for example, JY. The pooled fractions were HPLC fractions corresponding to 7% to 45% CH₃CN. For this class I molecule, this region of the chromatogram was most abundant in peptides. Data from independent experiments were averaged as described below.

The amino acid sequence analyses from four independent experiments were analyzed and the results are shown in Table 3. For each position except the first, the data were analyzed by modifying the method described by Falk et al., supra, to allow for comparison of experiments from different HLA types. This modified procedure yielded quantitative yet standardized values while allowing the averaging of data from different experiments involving the same HLA type.

The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation cycle) and 16 columns (each representing one of the twenty amino acids; W, C, R and H were eliminated for technical reasons. The data corresponding to the first row (first cycle) was not considered further because, this cycle is

usually heavily contaminated by free amino acids.). The values of each row were summed to yield a total pmoles value for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value, to determine what fraction of the total signal is attributable to each amino acid at each cycle. By doing so, an "Absolute Frequency" table was generated. This absolute frequency table allows correction for the declining yields of each cycle.

5

TABLE 3
A2.1: POOL SEQUENCING FREQUENCY

	pos. 1	pos. 2	pos. 3	pos. 4	pos. 5	pos. 6	pos. 7	pos. 8	pos. 9	pos. 10
A	-	0.65	1.25	0.85	0.95	0.77	1.21	1.16	1.15	1.25
G	-	0.84	0.96	1.29	1.22	0.89	0.78	1.05	0.98	1.48
D	-	0.84	1.11	1.70	1.03	0.83	0.82	0.84	0.82	1.19
E	-	0.38	0.59	1.73	1.10	0.82	1.05	1.45	0.87	0.88
R	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-
K	-	0.63	0.65	0.89	1.66	1.09	0.89	1.35	0.82	0.87
L	-	2.66*	1.11	0.45	0.57	1.00	0.69	0.59	0.92	0.77
V	-	0.78	0.69	0.60	0.79	1.38	1.24	0.84	1.69	1.27
I	-	1.06	1.20	0.53	0.93	1.49	1.15	0.76	0.88	0.54
M	-	1.93	1.91	0.62	0.71	0.68	0.88	0.54	0.73	0.22
Y	-	0.28	1.41	0.65	1.32	0.78	1.34	1.21	1.00	0.79
F	-	0.76	1.46	0.69	1.16	1.00	1.07	1.09	0.78	0.73
W	-	-	-	-	-	-	-	-	-	-
Q	-	0.60	0.84	0.92	0.95	0.90	1.16	1.63	1.00	1.00
N	-	0.39	0.76	1.17	1.28	1.08	1.07	1.28	0.96	0.42
S	-	1.13	1.50	1.33	0.87	0.77	0.71	0.92	0.77	0.58
T	-	0.62	0.90	0.94	0.95	1.21	1.07	1.60	0.71	0.57
C	-	-	-	-	-	-	-	-	-	-
P	-	0.54	0.78	1.44	1.15	1.09	1.30	0.87	0.81	1.01

* [REDACTED]

Starting from the absolute frequency table, a "relative frequency" table was then generated to allow comparisons among different amino acids. To do so the data from each column was summed, and then averaged. Then, each value was divided next by the average column value to obtain relative frequency values. These values quantitate, in a standardized manner, increases and decreases per cycle, for each of the different sixteen amino acid types. Tables generated from data from different experiments can thus be added together to generate average relative frequency values (and their standard deviations). All standard deviations can then be averaged, to estimate a standard deviation value applicable to the samples from each table. Any particular value exceeding 1.00 by more than two standard deviations is considered to correspond to a significant increase.

Example 4

Quantitative Binding Assays

Using isolated MHC molecules prepared as described in Example 2, above, quantitative binding assays were performed. Briefly, indicated amounts of MHC as isolated above were incubated in 0.05% NP40-PBS with ~5 nM of radiolabeled peptides in the presence of 1-3 μ M β_2 M and a cocktail of protease inhibitors (final concentrations 1 mM PMSF, 1.3 mM 1.10 Phenanthroline, 73 μ M Pepstatin A, 8 mM EDTA, 200 μ M N- α -p-tosyl-L-Lysine Chloromethyl ketone). After various times, free and bound peptides were separated by TSK 2000 gel filtration, as described previously in A. Sette et al., J. Immunol. 148:844 (1992), which is incorporated herein by reference. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

The HbC 18-27 peptide HLA binding peptide was radiolabeled and offered (5-10 nM) to 1 μ M purified HLA A2.1. After two days at 23°C in presence of a cocktail of protease inhibitors and 1-3 μ M purified human β_2 M, the percent of MHC class I bound radioactivity was measured by size exclusion chromatography, as previously described for class II peptide

binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Geftter, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202, which is incorporated herein by reference. Using this protocol, high binding (95%) was detected in all cases in the presence of purified HLA A2.1 molecules.

To explore the specificity of binding, we determined whether the binding was inhibitable by excess unlabeled peptide, and if so, what the 50% inhibitory concentration (IC50%) might be. The rationale for this experiment was threefold. First, such an experiment is crucial in order to demonstrate specificity. Second, a sensitive inhibition assay is the most viable alternative for a high throughput quantitative binding assay. Third, inhibition data subjected to Scatchard analysis can give quantitative estimates of the equilibrium constant (K) of interaction and the fraction of receptor molecules capable of binding ligand (% occupancy). For instance, in analysis of an inhibition curve for the interaction of the peptide HBC 18-27 with A2.1, the IC50% was determined to be 25 nM. Further experiments were conducted to obtain Scatchard plots. For HBC 18-27/A2.1, six different experiments using six independent MHC preparations yielded a K_D of $15.5 \pm 9.9 \times 10^{-9}$ M and occupancy values of 6.2% (± 1.4).

Several reports have demonstrated that class I molecules, unlike class II, are highly selective with regard to the size of the peptide epitope that they recognize. The optimal size varies between 8 and 10 residues for different peptides and different class I molecules, although MHC binding peptides as long as 13 residues have been identified. To verify the stringent size requirement, a series of N- and C-terminal truncation/extension analogs of the peptide HBC 18-27 were synthesized and tested for A2.1 binding. Previous studies had demonstrated that the optimal size for CTL recognition of this peptide was the 10-mer HBC18-27 (Sette et al. supra). It was found that removal or addition of a residue at the C terminus of the molecule resulted in a 30 to 100-fold decrease in binding capacity. Further removal or addition of another residue completely obliterated binding. Similarly, at the N-terminus of the molecule, removal or

deletion of one residue from the optimal HBC 18-27 peptide completely abrogated A2.1 binding.

Throughout this disclosure, results have been expressed in terms of IC₅₀'s. Given the conditions in which our assays are run (i.e., limiting MHC and labeled peptide concentrations), these values approximate K_D values. It should be noted that IC₅₀ values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (e.g., Class I preparation, etc.). For example, excessive concentrations of MHC will increase the apparent measured IC₅₀ of a given ligand.

An alternative way of expressing the binding data, to avoid these uncertainties, is as a relative value to a reference peptide. The reference peptide is included in every assay. As a particular assay becomes more, or less, sensitive, the IC₅₀'s of the peptides tested may change somewhat. However, the binding relative to the reference peptide will not change. For example, in an assay run under conditions such that the IC₅₀ of the reference peptide increases 10-fold, all IC₅₀ values will also shift approximately ten-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder should be based on it's IC₅₀, relative to the IC₅₀ of the standard peptide.

The reference peptide for the HLA-A2.1 assays described herein is referred to as 941.01 having a sequence of FLPSDYFPSV. An average IC₅₀ of 5 (nM) was observed under the assay conditions utilized.

If the IC₅₀ of the standard peptide measured in a particular assay is different from that reported in the table, then it should be understood that the threshold values used to determine good, intermediate, weak, and negative binders should be modified by a corresponding factor. For example, if in an A2.1 binding assay, the IC₅₀ of the A2.1 standard (941.01) were to be measured as 8 nM instead of 5 nM, then a peptide ligand would be called a good binder only if it had an

IC50 of less than 80 nM (i.e., 8nM x 0.1), instead of the usual cut-off value of 50 nM.

Example 5

HLA-A2.1 Binding Motif and Algorithm

The structural requirements for peptide binding to A2.1 have been defined for both, 9-mer and 10-mer peptides. Two approaches have been used. The first approach referred to as the "poly-A approach" uses a panel of single amino acid substitutions of a 9-mer prototype poly-A binder (ALAKAAAV) that is tested for A2.1 binding using the methods of Example 4 above to examine the degree of degeneracy of the anchor-positions and the possible influence of non-anchor positions on A2.1 binding.

The second approach, the "Motif-Library approach", uses a large library of peptides selected from sequences of potential target molecules of viral and tumor origin and tested for A2.1 binding using the methods in Example 4 above. The frequencies by which different amino-acids occurred at each position in good binders and non-binders were analysed to further define the role of non-anchor positions in 9-mers and 10-mers.

A2.1 binding of peptide 9-mers

Poly A Approach A poly-A 9-mer peptide, containing the A2.1 motif L (Leu) in position 2 and V (Val) in position 9 was chosen as a prototype binder. A K (Lys) residue was included in position 4 to increase solubility. A panel of 91 single amino-acid substitution analogues of the prototype parental 9-mer was synthesized and tested for A2.1 binding (Table 4). Shaded areas mark analogs with a greater than 10-fold reduction in binding capacity relative to the parental peptide. A reduction in binding greater than 100-fold is indicated by hyphenation.

Anchor-Positions 2 and 9 in poly-A Analogs The effect of single-amino-acid substitutions at the anchor positions 2 and 9 was examined first. Most substitutions in these positions had profound detrimental effects on binding

capacity, thus confirming their role for binding. More specifically, in position 2 only L and M bound within a 10-fold range ("preferred residues"). Residues with similar characteristics, such as I, V, A, and T were tolerated, but
5 bound 10 to 100-fold less strongly than the parental peptide. All the remaining substitutions (residues S, N, D, F, C, K, G, and P) were not tolerated and decreased binding by more than 100-fold. Comparably stringent requirements were observed for position 9, where V, L and I were preferred and A and M are
10 tolerated, while the residues T, C, N, F, and Y virtually abolished binding. According to this set of peptides, an optimal 2-9 motif could be defined with L, M in position 2 and V, I, or L in position 9.

TABLE 4
A2.1: BINDING OF ANALOGS OF A MOTIF-CONTAINING POLY A PEPTIDE

	pos. 1 A	pos. 2 L	pos. 3 A	pos. 4 K	pos. 5 A	pos. 6 A	pos. 7 A	pos. 8 A	pos. 9 V
A	1.00	0.013	1.00		1.00	1.00	1.00	1.00	0.070
G	0.46	-*			0.63	0.12		0.57	
D	-		0.93	0.74	0.51	0.10			
E	0.012		0.68	1.53	0.62	0.15	0.28	0.26	
R						0.080			
H								0.24	
K	0.54		0.062	1.00	0.39		0.50	0.11	0.11
L		1.00	0.46		0.99		0.76	0.90	1.00
V	0.47	0.051	0.15	1.12		0.44	0.49	0.30	0.18
I	0.41	0.063				1.12			0.024
M		0.43	0.66						
Y	0.75		0.62		0.94	0.41	1.40	0.43	-
F	1.10		0.95			1.76		0.49	-
W									
Q					0.32		0.19	0.41	
N			0.34		1.24		0.97	0.31	-
S	0.44		0.37	0.97					
T	0.26	0.011		0.98			0.28	0.37	-
C				1.53		0.84			-
P			0.25	1.07		0.84	0.63	0.55	

Ratio ≤ 0.1
Ratio ≤ 0.01

*

Non-Anchor Positions 1 and 3-8 in poly-A Analogs All

non-anchor positions were more permissive to different substitutions than the anchor-positions 2 and 9, i.e most residues were tolerated. Significant decreases in binding were observed for some substitutions in distinct positions. More specifically, in position 1 a negative charge (residues D and E) or a P greatly reduced the binding capacity. Most substitutions were tolerated in position 3 with the exception of the residue K. Significant decreases were also seen in position 6 upon introduction of either a negative charge (D, E) or a positively charged residue (R). A summary of these effects by different single amino acid substitutions is given in Table 5.

TABLE 5

Summary

A2.1

Poly-A

AA position	(+)	(+/-)	(-)
1	FAYKVGSI		EDP
2	LM	VITA	SNDFCKGP
3	AFDEMYLSNPV	K	
4	CEVPATSD		
5	NALYGEDKQ		
6	FIAPCVYEG	DR	
7	YANLPVETQ		
8	ALGPFYQTNVEHK		
9	VIL	AM	TCNFY

Ratio > 0.1

Ratio 0.01-0.1

Ratio < 0.01

The Motif-Library Approach To further evaluate the importance of non-anchor positions for binding, peptides of potential target molecules of viral and tumor origin were scanned for the presence of sequences containing optimal 2-9 anchor motifs. A set of 161 peptides containing a L or M in position 2 and a V, L or I in position 9 was selected, synthesized and tested for binding (see Example 6). Only 11.8% of these peptides bind with high affinity (ratio ≥ 0.10 ; 22.4% were intermediate binders (ratio ≥ 0.1). As many as 36% were weak binders (ratio $< 0.01 - 0.0001$), and 31% were non-binders (ratio < 0.0001). The high number of non-binders containing optimal anchor-motifs indicates that in this set of peptides positions other than the 2-9 anchors influence A2.1 binding capacity. Appendix 1 sets forth all of the peptides having the 2-9 motif used for this analysis and the binding data for those peptides.

To define the influence on non-anchor positions more specifically, the frequency of occurrence of each amino acid

in each of the non-anchor positions was calculated for the good and intermediate binders on one hand and non-binders on the other hand. Amino acids of similar chemical characteristic were grouped together. Weak binders were not considered for the following analysis. The frequency of occurrence of each amino acid in each of the non-anchor positions was calculated for the good binders and non-binders (Table 6).

Several striking trends become apparent. For example in position 1, only 3.6% of the A2.1 binders and as much as 35% of the non-binders carried a negative charge (residues D and E). This observation correlates well with previous findings in the set of poly-A analogs, where a D or E substitution greatly affected binding. Similarly, the residue P was 8 times more frequent in non-binders than in good binders. Conversely, the frequencies of aromatic residues (Y, F, W) were greatly increased in A2.1 binders as compared to non-binders.

TABLE 6

A.2.1 9-mer peptides	161	11.8%
NUMBER OF PEPTIDES	19	22.4%
GOOD BINDERS	36	36.0%
INTERMEDIATE BINDERS	58	29.8%
WEAK BINDERS	48	
NON-BINDERS		

[illegible]

Following this approach, amino acids of similar structural characteristics were grouped together. Then, the frequency of each amino acid group in each position was calculated for binders versus non-binders (Table 7). Finally, the frequency in the binders group was divided by the frequency in the non-binders to obtain a "frequency ratio". This ratio indicates whether a given amino-acid or group of residues occurs in a given position preferentially in good binders (ratio >1) or in non-binders (ratio <1).

TABLE 7

A2.1 9-mer PEPTIDES

NUMBER OF PEPTIDES	161
GOOD BINDERS	19 11.8%
INTERMEDIATE BINDERS	36 22.4%
WEAK BINDERS	58 36.0%
NON-BINDERS	48 29.8%

	pos. 1 ratio	pos. 2 ratio	pos. 3 ratio	pos. 4 ratio	pos. 5 ratio	pos. 6 ratio	pos. 7 ratio	pos. 8 ratio	pos. 9 ratio
A	2.6	NA	0.9	0.9	0.7	0.9	4.4	0.3	NA
G	3.5	NA	0.4	1.1	1.1	1.3	0.4	0.4	NA
D,E	0.1	NA	0.0	0.7	0.3	0.7	0.1	0.9	NA
R,H,K	3.1	NA	0.2	1.0	0.9	0.1	0.0	1.3	NA
L,V,I,M	3.1	1.0	1.8	0.5	0.9	1.3	1.2	1.7	1.0
Y,F,W	7.0	NA	5.2	0.9	8.7	2.0	2.3	2.6	NA
Q,N	0.5	NA	0.4	1.2	0.9	1.0	0.7	0.3	NA
S,T,C	0.7	NA	1.9	4.8	0.9	1.2	1.2	1.1	NA
P	0.1	NA	0.7	0.7	2.6	1.7	2.9	+++	NA

+++ indicates that there were no negative binders

Different Residues Influence A2.1 Binding In order to analyse the most striking influences of certain residues on A2.1 binding, a threshold level was set for the ratios described in Table 7. Residues showing a more than 4-fold greater frequency in good binders were regarded as preferred residues (+). Residues showing a 4-fold lower frequency in A2.1 binders than in non-binders were regarded as disfavored residues (-). Following this approach, residues showing the most prominent positive or negative effects on binding are listed in Table 8.

This table identifies the amino acid groups which influence binding most significantly in each of the non-anchor positions. In general, the most negative effects were observed with charged amino acids. In position 1, negatively, charged amino acids were not observed in good binders, i.e., those amino acids were negative binding residues at position 1. The opposite was true for position 6 where only basic amino acids were detrimental for binding i.e., were negative binding residues. Moreover, both acidic and basic amino acids were not observed in A2.1 binders in positions 3 and 7. A greater than 4-fold increased frequency of non-binders was found when P was in position 1.

TABLE 8

Summary of A2.1 Motif-Library, 9-mers

AA POSITION	(+)	(-)
1	(YFW)	P, (DE)
2	Anchor	
3	(YFW)	(DE), (RKH)
4	(STC)	
5	(YFW)	
6		(RKH)
7	A	(RKH), (DE)
8		
9	Anchor	

(+) = Ratio \geq 4-fold (-) = Ratio \leq 0.25

Aromatic residues were in general favored in several of the non-anchor positions, particularly in positions 1, 3, and 5. Small residues like S, T, and C were favored in position 4 and A was favored in position 7.

An Improved A2.1 9-mer Motif The data described above was used to derive a stringent A2.1 motif. This motif is based in significant part on the effects of the non-anchor positions 1 and 3-8. The uneven distribution of amino acids at different positions is reflective of specific dominant negative binding effects of certain residues, mainly charged

ones, on binding affinity. A series of rules were derived to identify appropriate anchor residues in positions 2 and 9 and negative binding residues at positions 1 and 3-8 to enable selection of a high affinity binding immunogenic peptide.

5 These rules are summarized in Table 9.

To validate the motif defined above and shown in Table 9 published sequences of peptides that have been naturally processed and presented by A2.1 molecules were analysed (Table 10). Only 9-mer peptides containing the 2-9 anchor residues
10 were considered.

When the frequencies of these peptides were analysed, it was found that in general they followed the rules summarized in Table 9. More specifically, neither acidic amino acids nor P were found in position 1. Only one acidic amino acid and no
15 basic amino acids were found in position 3. Positions 6 and 7 showed no charged residues. Acidic amino acids, however, were frequently found in position 8, where they are tolerated, according to our definition of the A2.1 motif. The analysis of the sequences of naturally processed peptides therefore
20 reveals that >90% of the peptides followed the defined rules for a complete motif.

Thus the data confirms a role of positions other than the anchor positions 2 and 9 for A2.1 binding. Most of the deleterious effects on binding are induced by charged amino
25 acids in non-anchor positions, i.e. negative binding residues occupying positions 1, 3, 6 or 7.

TABLE 9
A2.1 MOTIF FOR 9-MER PEPTIDES

AA Position	(+)	(-)
1		acidic amino-acids and P
2	Anchor: L, M, (I,V,A,T)	
3		acidic and basic amino-acids
4		
5		
6		basic amino-acids
7		acidic and basic amino-acids
8		
9	Anchor: V, I, L (A,M)	

TABLE 10

A2.1 naturally processed peptides

1	2	3	4	5	6	7	8	9	A2.1 binding
A	L	X	G	G	X	V	N	V	ND
L	L	D	V	P	T	A	A	V	ND
G	X	V	P	F	X	V	S	V	0.41
S	L	L	P	A	I	V	E	L	0.19
S	X	X	V	R	A	X	E	V	ND
Y	M	N	G	T	M	S	Q	V	ND
K	X	N	E	P	V	X	X	X	ND
Y	L	L	P	A	I	V	H	I	0.26
A	X	W	G	F	F	P	V	X	ND
T	L	W	V	D	P	Y	E	V	0.23
G	X	V	P	F	X	V	S	V	0.41

A2.1 Binding of Peptide 10-mers

The "Motif-Library" Approach Previous data clearly indicated that 10-mers can also bind to HLA molecules even if with a somewhat lower affinity than 9-mers. For this reason we expanded our analysis to 10-mer peptides.

Therefore, a "Motif-Library" set of 170 peptide 10-mers containing optimal motif-combinations was selected from known target molecule sequences of viral and tumor origin and analysed as described above for 9-mers. In this set we found 5.9% good binders, 17.1% intermediate binders, 41.2% weak binders and 35.9% non-binders. The actual sequences, origin and binding capacities of this set of peptides are included as Appendix 2. This set of 10-mers was used to determine a) the rules for 10-mer peptide binding to A2.1, b) the similarities or differences to rules defined for 9-mers, and c) if an insertion point can be identified that would allow for a superimposable common motif for 9-mers and 10-mers.

Amino-acid frequencies and frequency ratios for the various amino-acid groups for each position were generated for 10-mer peptides as described above for 9-mer peptides and are also shown in Tables 11 and 12, respectively for grouped residues.

A summary of preferred versus disfavored residues and of the rules derived for the 10-mers in a manner analogous to that used for 9-mers, is also listed in Tables 13 and 14, respectively.

When the frequency-ratios of different amino-acid groups in binders and non-binders at different positions were analysed and compared to the corresponding ratios for the 9-mers, both striking similarities and significant differences emerged (Table 15). At the N-terminus and the C-termini of 9-mers and 10-mers, similarities predominate. In position 1 for example, in 10-mers again the P residue and acidic amino acids were not tolerated. In addition at position 1 in 10-mers aromatic residues were frequently observed in A2.1 binders. In position 3, acidic amino acids were frequently associated with poor binding capacity in both 9-mers and 10-mers. Interestingly, however, while in position 3 aromatic residues

TABLE 12

A2.1 10-mer Peptides

NUMBER OF PEPTIDES
 GOOD BINDERS 170 5.9%
 INTERMEDIATE BINDERS 29 17.1%
 WEAK BINDERS 70 41.2%
 NON-BINDERS 61 35.9%

	pos. 1 ratio	pos. 2 ratio	pos. 3 ratio	pos. 4 ratio	pos. 5 ratio	pos. 6 ratio	pos. 7 ratio	pos. 8 ratio	pos. 9 ratio	pos. 10 ratio
A	+++	NA	3.1	0.2	1.8	0.6	1.3	1.6	0.5	NA
G	0.8	NA	0.5	4.7	0.8	6.3	2.7	0.7	0.8	NA
D,E	0.0	NA	0.2	0.6	0.3	1.0	0.3	0.0	0.4	NA
R,H,K	1.2	NA	0.3	0.1	0.7	0.4	0.2	0.0	0.2	NA
L,V,I,M	3.0	1.0	10.2	1.0	1.3	2.1	1.4	4.7	0.8	1.0
Y,F,W	+++	NA	2.6	3.1	3.6	0.6	1.6	14.1	2.1	NA
Q,N	1.0	NA	0.9	0.8	0.8	0.8	0.6	0.4	0.7	NA
S,T,C	0.9	NA	0.9	1.1	1.0	0.9	1.4	1.3	2.9	NA
P	0.0	NA	0.4	2.6	0.0	1.0	0.4	1.9	1.2	NA

+++ Indicates that there were no negative binders.

TABLE 13
Summary of A2.1 Motif-Library 10-mers

AA position	(+)	(-)
1	(YFW), A	(DE), P
2	Anchor	
3	(LVIM)	(DE)
4	G	A, (RKH)
5		P
6	G	
7		(RKH)
8	(YFW), (LVIM)	(DE), (RKH)
9		(RKH)
10	Anchor	

(+) = Ratio \geq 4-fold

(-) = Ratio \leq 0.25

TABLE 14

A2.1 MOTIF FOR 10-MER PEPTIDES

AA Position	(+)	(-)
1		acidic amino-acids and P
2	Anchor: L, M, (I, V, A, T)	
3		acidic amino-acids
4		basic amino-acids and A
5		P
6		
7		basic amino-acids
8		acidic and basic amino- acids
9		basic amino-acids
10	Anchor: V, I, L (A, M)	

TABLE 15
COMPARISON OF A2.1 BINDING OF 9-MERS AND 10-MERS

AA Position	9-mers (+)	10-mers (+)
1	(YFW)	(YFW)
2	Anchor	Anchor
3	(YWF)	(LVIM)
4	(STC)	G
5	(YWF)	
6		G
7	A	
8		(YWF), (LVIM)
9	Anchor	
10	-	Anchor

AA Position	9-mers (-)	10-mers (-)
1	P, (DE)	P, (DE)
2	Anchor	Anchor
3	(DE), (RKH)	(DE)
4		A, (RKH)
5		P
6	(RKH)	
7	(DE), (RKH)	(RKH)
8		(DE), (RKH)
9	Anchor	(RKH)
10	-	Anchor

were preferred in 9-mers, aliphatic residues (L, V, I, M) were preferred in 10-mers.

At the C-terminus of the peptides, basic amino acids are not favored in position 7, and both acidic and basic amino acids are not favored in position 8 for 10-mers. This is in striking agreement with the observation that the same pattern was found in 9-mers at positions 6 and 7. Interestingly, again the favored residues differ between two peptides sizes.

Aromatic (Y, F, W) or aliphatic (L, V, I, M) residues were preferred in 10-mers at position 8, while the A residue was preferred by 9-mers in the corresponding position 7.

By contrast, in the center of the peptide no similarities of frequency preferences were observed at positions 4, 5, and 6 in 10-mers and positions 4 and 5 in the 9-mers.

Most interestingly, among the residues most favored in the center of the tested peptides were G in position 4 and 6, P in position 5 was not observed in binders. All of these residues are known to dramatically influence the overall secondary structure of peptides, and in particular would be predicted to strongly influence the propensity of a 10-mer to adopt a "kinked" or "bulged" conformation.

Charged residues are predominantly deleterious for binding and are frequently observed in non-binders of 9-mers and 10-mers.

However, favored residues are different for 9-mers and 10-mers. Glycine is favored while Proline is disfavored in the center of 10-mer peptides but this is not the case for 9-mers.

These data establish the existence of an "insertion area" spanning two positions (4, 5) in 9-mers and 3 positions (4, 5, 6) in 10-mers. This insertion area is a more permissive region where few residue similarities are observed between the 9-mer and 10-mer antigenic peptides. Furthermore, in addition to the highly conserved anchor positions 2 and 9, there are "anchor areas" for unfavored residues in positions 1 and 3 at the N-terminus for both 9-mer and 10-mer and

positions 7-10 or 6-9 at the C-terminus for 10-mers and 9-mers, respectively.

Example 6

5 Algorithm to Predict Binding of 9-mer Peptides to HLA-A2.1

Within the population of potential A2.1 binding peptides identified by the 2,9 motif, as shown in the previous example, only a few peptides are actually good or intermediate binders and thus potentially immunogenic. It is apparent from
10 the data previously described that the residues present in positions other than 2 and 9 can influence, often profoundly, the binding affinity of a peptide. For example, acidic residues at position 1 for A2.1 peptides do not appear to be tolerated. Therefore, a more exact predictor of binding could
15 be generated by taking into account the effects of different residues at each position of a peptide sequence, in addition to positions 2 and 9.

More specifically, we have utilized the data bank obtained during the screening of our collection of A2.1 motif
20 containing 9-mer peptides to develop an algorithm which assigns a score for each amino acid, at each position along a peptide. The score for each residue is taken as the ratio of the frequency of that residue in good and intermediate binders to the frequency of occurrence of that residue in non-binders.

25 In the present "Grouped Ratio" algorithm residues have been grouped by similarity. This avoids the problem encountered with some rare residues, such as tryptophan, where there are too few occurrences to obtain a statistically significant ratio. Table 16 is a listing of scores obtained
30 by grouping for each of the twenty amino acids by position for 9-mer peptides containing perfect 2/9 motifs. A peptide is scored in the "Grouped Ratio" algorithm as a product of the scores of each of its residues. In the case of positions
35 other than 2 and 9, the scores have been derived using a set of peptides which contain only preferred residues in positions 2 and 9. To enable us to extend our "Grouped Ratio" algorithm

TABLE 16

	1	2	3	4	5	6	7	8	9
A	2.6	0.03	0.87	0.87	0.65	0.87	4.4	0.29	0.16
C	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
D	0.10	0.01	0.10	0.65	0.29	0.65	0.11	0.87	0.01
E	0.10	0.01	0.10	0.65	0.29	0.65	0.11	0.87	0.01
F	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01
G	3.5	0.01	0.44	1.1	1.1	1.3	0.44	0.44	0.01
H	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
I	3.1	0.14	1.8	0.55	0.87	1.4	1.2	1.8	0.40
K	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
L	3.1	1.00	1.8	0.55	0.87	1.4	1.2	1.8	0.09
M	3.1	2.00	1.8	0.55	0.87	1.4	1.2	1.8	0.06
N	0.50	0.01	0.37	1.2	0.87	1.1	0.65	0.33	0.01
P	0.12	0.01	0.70	0.73	2.6	1.8	2.9	0.10	0.01
Q	0.50	0.01	0.37	1.2	0.87	1.1	0.65	0.33	0.01
R	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
S	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
T	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
V	3.1	0.08	1.8	0.55	0.87	1.4	1.2	1.8	1.00
W	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01
Y	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01

to peptides which may have residues other than the preferred ones at 2 and 9, scores for 2 and 9 have been derived from a set of peptides which are single amino acid substitutions at positions 2 and 9. Figure 2 shows a scattergram of the log of relative binding plotted against "Grouped Ratio" algorithm score for our collection of 9-mer peptides from the previous example.

The present "Grouped Ratio" algorithm can be used to predict a population of peptides with the highest occurrence of good binders. If one were to rely, for example, solely on a 2(L,M) and 9(V) motif for predicting A2.1 binding 9-mer peptides, it would have been predicted that all 160 peptides in our database would be good binders. In fact, as has already been described, only 12% of these peptides would be described as good binders and only 22% as intermediate binders; 66% of the peptides predicted by such a 2,9 motif are either weak or non-binding peptides. In contrast, using the "Grouped Ratio" algorithm described above, and selecting a score of 1.0 as threshold, 41 peptides were selected. Of this set, 27% are good binders, and 49% are intermediate, while only 20% are weak and 5% are non-binders (Table 17).

The present example of an algorithm has used the ratio of binders/non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill that there are alternative ways of creating a similar algorithm.

An algorithm using the average binding affinity of all the peptides with a certain amino acid (or amino acid type) at a certain position has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler "Grouped Ratio" algorithm. We have created such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of 160 2,9 motif containing peptides. These values are shown in Table 18. The algorithm score for a peptide is then taken as the sum of the scores by position for each residues.

Figure 3 shows a scattergram of the log of relative binding against the average "Log of Binding" algorithm score. Table 17 shows the ability of the two algorithms to predict peptide binding at various levels, as a function of the cut-off score used. The ability of a 2,9 motif to predict binding in the same peptide set is also shown for reference purposes. It is clear from this comparison that both algorithms of this invention have a greater ability to predict populations with higher frequencies of good binders than a 2,9 motif alone. Differences between the "Grouped Ratio" algorithm and the "Log of Binding" algorithm are small in the set of peptides analyzed here, but do suggest that the "Log of Binding" algorithm is a better, if only slightly, predictor than the "Grouped Ratio" algorithm.

The log of binding algorithm was further revised in two ways. First, poly-alanine (poly-A) data were incorporated into the algorithms at the anchor positions for residues included in the expanded motifs where data obtained by screening a large library of peptides were not available. Second, an "anchor requirement screening filter" was incorporated into the algorithm. The poly-A approach is described in detail, above. The "anchor requirement screening filter" refers to the way in which residues are scored at the anchor positions, thereby providing the ability to screen out peptides which do not have preferred or tolerated residues in the anchor positions. This is accomplished by assigning a score for unacceptable residues at the anchor positions which are so high as to preclude any peptide which contains them from achieving an overall score which would allow it to be considered as a potential binder.

The results for 9-mers and 10-mers are presented in Tables 26 and 27, below. In these tables, values are group values as follows: A; G; P; D,E; R,H,K; L,I,V,M; F,Y,W; S,T,C; and Q,N, except where noted in the tables.

TABLE 17

Criteria	Cut-off	Good Binders	Intermediate Binders	Weak Binders	Negative Binders	Totals
2.9 motif		19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)
Grouped Ratio						
Algorithm	1.5	5 (83%)	1 (17%)	0 (0%)	0 (0%)	6 (100%)
	1.25	8 (67%)	4 (33%)	0 (0%)	0 (0%)	12 (100%)
	1	10 (50%)	9 (45%)	1 (5%)	0 (0%)	20 (100%)
	0.5	12 (32%)	17 (46%)	7 (19%)	1 (3%)	37 (100%)
	0	12 (23%)	26 (49%)	12 (23%)	3 (6%)	53 (100%)
	-1	17 (18%)	35 (37%)	33 (35%)	10 (11%)	95 (100%)
	-2	19 (15%)	36 (29%)	50 (40%)	21 (17%)	126 (100%)
	-3	19 (13%)	36 (24%)	56 (38%)	38 (26%)	149 (100%)
	no cut	19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)
Log of Binding						
Algorithm	-19	5 (100%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)
	-20	8 (73%)	3 (27%)	0 (0%)	0 (0%)	11 (100%)
	-21	15 (43%)	15 (43%)	5 (14%)	0 (0%)	35 (100%)
	-22	17 (26%)	27 (41%)	21 (32%)	1 (2%)	68 (100%)
	-23	18 (19%)	35 (37%)	34 (36%)	7 (7%)	94 (100%)
	-24	18 (16%)	36 (30%)	47 (39%)	17 (14%)	119 (100%)
	-25	19 (14%)	36 (26%)	55 (39%)	30 (21%)	140 (100%)
	no cut	19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)

TABLE 18

	1	2	3	4	5	6	7	8	9
A	-2.38	-3.22	-2.80	-2.68	-2.89	-2.70	-2.35	-3.07	-2.49
C	-2.94	-4.00	-2.58	-1.96	-3.29	-2.22	-2.97	-2.37	-4.00
D	-3.69	-4.00	-3.46	-2.71	-2.26	-2.63	-3.61	-3.03	-4.00
E	-3.64	-4.00	-3.51	-2.65	-3.39	-3.41	-3.21	-2.63	-4.00
F	-1.89	-4.00	-2.35	-2.50	-1.34	-2.43	-2.18	-1.71	-4.00
G	-2.32	-4.00	-3.04	-2.63	-2.56	-2.30	-3.13	-2.96	-4.00
H	-2.67	-4.00	-2.58	-2.58	-2.05	-3.32	-3.13	-2.16	-4.00
I	-1.65	-2.55	-2.80	-3.44	-2.74	-2.79	-2.20	-2.69	-2.10
K	-2.51	-4.00	-3.65	-2.93	-3.34	-3.77	-3.13	-3.27	-4.00
L	-2.32	-1.70	-2.02	-2.49	-2.71	-2.63	-2.62	-2.01	-2.74
M	-0.39	-1.39	-1.79	-3.07	-3.43	-1.38	-1.33	-0.97	-2.96
N	-3.12	-4.00	-3.52	-2.22	-2.36	-2.30	-3.14	-3.31	-4.00
P	-3.61	-4.00	-2.97	-2.64	-2.42	-2.31	-1.83	-2.42	-4.00
Q	-2.76	-4.00	-2.81	-2.63	-3.06	-2.84	-2.12	-3.05	-4.00
R	-1.92	-4.00	-3.41	-2.61	-3.05	-3.76	-3.43	-3.02	-4.00
S	-2.39	-3.52	-2.04	-2.12	-2.83	-3.04	-2.73	-2.02	-4.00
T	-2.92	-4.00	-2.60	-2.48	-2.17	-2.58	-2.67	-3.14	-3.70
V	-2.44	-2.64	-2.68	-3.29	-2.49	-2.24	-2.68	-2.83	-1.70
W	-0.14	-4.00	-1.01	-2.94	-1.63	-2.77	-2.85	-2.13	-4.00
X	-1.99	-2.13	-2.41	-2.97	-2.72	-2.70	-2.41	-2.35	-2.42
Y	-1.46	-4.00	-1.67	-2.70	-1.92	-2.39	-1.35	-3.37	-4.00

Example 7Use of an Algorithm to Predict Binding of 10-mer Peptides to
HLA-A2.1

5 Using the methods described in the proceeding
example, an analogous set of algorithms has been developed for
predicting the binding of 10-mer peptides. Table 19 shows the
scores used in a "Grouped Ratio" algorithm, and Table 20 shows
the "Log of Binding" algorithm scores, for 10-mer peptides.
10 Table 21 shows a comparison of the application of the two
different algorithmic methods for selecting binding peptides.
Figures 4 and 5 show, respectively, scattergrams of a set of
10-mer peptides containing preferred residues in positions 2
and 10 as scored by the "Grouped Ratio" and "Log of Binding"
15 algorithms.

TABLE 19

	1	2	3	4	5	6	7	8	9	10
A	3.00	0.01	3.10	0.20	1.60	0.60	1.30	1.60	0.50	0.01
C	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
D	0.01	0.01	0.20	0.60	0.30	1.00	0.30	0.01	0.40	0.01
E	0.01	0.01	0.20	0.60	0.30	1.00	0.30	0.01	0.40	0.01
F	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01
G	0.80	0.01	0.50	4.70	0.80	6.30	2.70	0.70	0.80	0.01
H	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
I	3.00	0.50	10.2	1.00	1.30	2.10	1.40	4.70	0.80	1.00
K	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
L	3.00	1.10	10.2	1.00	1.30	2.10	1.40	4.70	0.80	0.50
M	3.00	0.60	10.2	1.00	1.30	2.10	1.40	4.70	0.80	0.01
N	1.00	0.01	0.90	0.80	0.80	0.80	0.60	0.40	0.70	0.01
P	0.00	0.01	0.40	2.60	0.01	1.00	0.40	1.90	1.20	0.01
Q	1.00	0.01	0.90	0.80	0.80	0.80	0.60	0.40	0.70	0.01
R	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
S	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
T	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
V	3.00	0.10	10.2	1.00	1.30	2.10	1.40	4.70	0.80	2.30
W	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01
Y	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01

TABLE 20

	1	2	3	4	5	6	7	8	9	10
A	-2.40	-4.00	-2.54	-3.42	-3.07	-3.30	-2.98	-2.69	-3.29	-4.00
C	-3.64	-4.00	-2.47	-2.48	-1.78	-3.94	-1.28	-3.10	-2.43	-4.00
D	-3.65	-4.00	-2.76	-3.26	-2.76	-3.03	-3.43	-3.68	-3.63	-4.00
E	-3.92	-4.00	-3.63	-3.34	-3.73	-2.82	-3.54	-3.71	-2.95	-4.00
F	-1.52	-4.00	-1.96	-3.03	-2.01	-3.11	-2.67	-1.61	-2.43	-4.00
G	-2.91	-4.00	-3.40	-2.63	-2.98	-2.45	-2.52	-3.18	-3.03	-4.00
H	-3.61	-4.00	-3.10	-3.03	-2.33	-2.99	-3.70	-3.55	-4.00	-4.00
I	-2.26	-4.00	-2.82	-3.05	-2.38	-2.61	-2.38	-3.34	-3.18	-1.47
K	-2.53	-4.00	-3.65	-3.42	-3.14	-3.58	-3.50	-3.53	-4.00	-4.00
L	-2.00	-2.93	-2.21	-2.48	-2.88	-2.53	-2.57	-1.83	-3.23	-3.20
M	-2.41	-3.11	-2.00	-3.33	-3.70	-2.56	-3.27	-2.25	-3.00	-4.00
N	-3.21	-4.00	-3.09	-2.61	-2.93	-2.89	-3.52	-3.01	-2.88	-4.00
P	-3.90	-4.00	-3.21	-2.27	-3.72	-3.06	-3.35	-2.58	-2.94	-4.00
Q	-2.92	-4.00	-2.97	-4.00	-2.98	-3.46	-2.20	-3.23	-3.45	-4.00
R	-3.01	-4.00	-3.44	-3.50	-3.23	-3.32	-3.72	-3.59	-2.97	-4.00
S	-2.47	-4.00	-3.17	-3.11	-3.23	-2.64	-3.19	-2.79	-2.26	-4.00
T	-3.59	-4.00	-3.07	-2.88	-2.89	-3.16	-2.43	-3.11	-2.58	-4.00
V	-2.97	-4.00	-2.46	-3.14	-3.27	-2.53	-3.14	-3.02	-2.90	-2.61
W	-2.10	-4.00	-2.72	-1.79	-2.65	-1.92	-1.80	-2.24	-2.11	-4.00
Y	-2.37	-4.00	-2.42	-2.85	-3.03	-3.76	-2.82	-2.34	-2.74	-4.00

TABLE 21

Criteria	Cut-off	Good	Intermediate	Weak	Negative	Totals
2,10 motif		10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)
Grouped Ratio	4	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Algorithm	3	1 (25%)	2 (50%)	1 (25%)	0 (0%)	4 (100%)
	2	6 (24%)	13 (52%)	6 (24%)	0 (0%)	25 (100%)
	1	10 (21%)	21 (45%)	16 (34%)	0 (0%)	47 (100%)
	0	10 (15%)	28 (42%)	26 (39%)	2 (3%)	66 (100%)
	-1	10 (11%)	29 (32%)	42 (46%)	11 (12%)	92 (100%)
	-2	10 (9%)	29 (25%)	54 (47%)	23 (20%)	116 (100%)
	-3	10 (7%)	29 (22%)	63 (47%)	32 (24%)	134 (100%)
	no cut	10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)
Log of Binding	-24	2 (50%)	2 (50%)	0 (0%)	0 (0%)	4 (100%)
Algorithm	-25	5 (56%)	3 (33%)	1 (11%)	0 (0%)	9 (100%)
	-26	7 (47%)	5 (33%)	3 (20%)	0 (0%)	15 (100%)
	-27	10 (32%)	9 (29%)	12 (39%)	0 (0%)	31 (100%)
	-28	10 (17%)	19 (33%)	29 (50%)	0 (0%)	58 (100%)
	-29	10 (12%)	25 (30%)	48 (58%)	0 (0%)	83 (100%)
	-30	10 (10%)	29 (28%)	59 (57%)	5 (5%)	103 (100%)
	-31	10 (8%)	28 (22%)	66 (51%)	24 (19%)	129 (100%)
	-32	10 (7%)	29 (19%)	70 (47%)	40 (27%)	149 (100%)
	no cut	10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)

Example 8Binding of A2.1 Algorithm Predicted Peptides

5 The results of Examples 6 and 7 indicate that an algorithm can be used to select peptides that bind to HLA-A2.1 sufficiently to have a high probability of being immunogenic.

To test this result, we tested our algorithm on a large (over 1300) non-redundant, independent set of peptides derived from various sources. After scoring this set with our algorithm,
10 we selected 41 peptides (Table 21) for synthesis, and tested them for A2.1 binding. This set of peptides was comprised of 21 peptides with high algorithm scores, and 20 peptides with low algorithm scores.

The binding data and categorization profile are shown in
15 Tables 22 and 23 respectively. The correlation between binding and algorithm score was 0.69. It is immediately apparent from Table 23 the striking difference between peptides with high algorithm scores, and those with low algorithm scores. Respectively, 76% of the high scorers and
20 none of the low scorers were either good or intermediate binders. This data demonstrates the utility of the algorithm of this invention.

TABLE 22

SEQUENCE	SOURCE	A2.1 Binding	Algorithm Score
MMWFFVLTIV	CMV	0.76	346
YLLLYFSPV	CMV	0.75	312
YLRLNFCL	CMV	0.72	169
FMWTYLVTL	CMV	0.68	336
LLWWITILL	CMV	0.49	356
GLWCVLFFV	CMV	0.47	1989
LMIRGVLEV	CMV	0.45	296
LLLCRLPFL	CMV	0.42	1356
RLLTSLFPL	HSV	0.34	859
LLLYDYDSL	HSV	0.28	390
AMSRNLFRV	CMV	0.15	1746
AMLTACVEV	CMV	0.089	411
RLQPNVPLV	CMV	0.048	392
VLARTFTPV	CMV	0.044	196
RLLRGURL	CMV	0.037	494
WMWFPSVLL	CMV	0.036	362
YLCCGITLL	CMV	0.021	1043
DMLGRVFFV	HSV	0.011	1422
ALGRYQQLV	CMV	0.0089	184
LMPPPVAEL	CMV	0.0066	416
LMCRYTPRL	CMV	0.0055	414
RLTWRLTWL	CMV	0.0052	250
AMPRRVLHV	CMV	0.0014	628
ALLLVLALL	CMV	0.0014	535
AMSGTGTTL	CMV	0.0005	602
MLNVMKEAV	CMV	0.0039	0.00031
TMELMIRTV	CMV	0.0029	0.0013
TLAAMHSL	HSV	0.0008	0.0019
TLNIVRDHV	CMV	0.0005	0.00021
ELSI FRERL	HSV	0.0002	0.0020
FLRVQQKAL	HSV	0.0002	0.00099
ELQMMQDWV	CMV	0.0001	0.0020
QLNAMKPD	MT	0.0001	0.0017
GLRQLKGAL	CMV	0.0001	0.0010
TLRMSSKAV	HSV	0.0001	0.00085
SLRIKRELL	CMV	0	0.00041
DLKQMERVV	CMV	0	0.00026
PLRVTPSDL	CMV	0	0.0019
QLDYEKQVL	CMV	0	0.0012
WLKLLRDAL	CMV	0	0.0012
PMEAVRHPL	CMV	0	0.0011
ELKQTRVNL	CMV	0	0.00053
NLEVIHDAL	CMV	0	0.00050
ELKKVKSVL	HSV	0	0.00033
PLAYERDKL	CMV	0	0.00017

TABLE 23

Set	Good Binders	Intermediate Binders	Weak Binders	Negative Binders	Totals
HI Scorers	11 (52.4%)	5 (23.8%)	5 (23.8%)	0 (0.0%)	21 (100%)
Low Scorers	0 (0.0%)	C (0.0%)	10 (50.0%)	10 (50.0%)	20 (100%)
Totals	11 (26.6%)	5 (12.2%)	15 (36.6%)	10 (24.4%)	41 (100%)

Example 9

Ex vivo induction of Cytotoxic T Lymphocytes (CTL)

Peripheral blood mononuclear cells (PBMC) are isolated from an HLA-typed patient by either venipuncture or apheresis (depending upon the initial amount of CTLp required), and purified by gradient centrifugation using Ficoll-Paque (Pharmacia). Typically, one can obtain one million PBMC for every ml of peripheral blood, or alternatively, a typical apheresis procedure can yield up to a total of $1-10 \times 10^{10}$ PBMC.

The isolated and purified PBMC are co-cultured with an appropriate number of antigen presenting cell (APC), previously incubated ("pulsed") with an appropriate amount of synthetic peptide (containing the HLA binding motif and the sequence of the antigen in question). PBMC are usually incubated at $1-2 \times 10^6$ cells/ml in culture medium such as RPMI-1640 (with autologous serum or plasma) or the serum-free medium AIM-V (Gibco).

APC are usually used at concentrations ranging from 1×10^4 to 2×10^5 cells/ml, depending on the type of cell used. Possible sources of APC include: 1) autologous dendritic cells (DC), which are isolated from PBMC and purified as described (Inaba, et al., J. Exp. Med. 166:182 (1987)); and 2) mutant and genetically engineered mammalian cells that express "empty" HLA molecules (which are syngeneic [genetically identical] to the patient's allelic HLA form), such as the, mouse RMA-S cell line or the human T2 cell line. APC containing empty HLA molecules are known to be potent inducers of CTL responses, possibly because the peptide can associate more readily with empty MHC molecules than with MHC molecules which are occupied by other peptides (DeBruijn, et al., Eur. J. Immunol. 21:2963-2970 (1991)).

In those cases when the APC used are not autologous, the cells will have to be gamma irradiated with an appropriate dose (using, e.g., radioactive cesium or cobalt) to prevent their proliferation both ex vivo, and when the cells are re-introduced into the patients.

The mixture cultures, containing PBMC, APC and peptide are kept in an appropriate culture vessel such as plastic T-flasks, gas-permeable plastic bags, or roller bottles, at 37° centigrade in a humid air/CO₂ incubator.

5 After the activation phase of the culture, which usually occurs during the first 3-5 days, the resulting effector CTL can be further expanded, by the addition of recombinant DNA-derived growth factors such as interleukin-2 (IL-2), interleukin-4 (IL-4), or interleukin-7 (IL-7) to the cultures.

10 An expansion culture can be kept for an additional 5 to 12 days, depending on the numbers of effector CTL required for a particular patient. In addition, expansion cultures may be performed using hollow fiber artificial capillary systems (Cellco), where larger numbers of cells (up to 1×10^{11}) can be

15 maintained.

Before the cells are infused into the patient, they are tested for activity, viability, toxicity and sterility. The cytotoxic activity of the resulting CTL can be determined by a standard ⁵¹Cr-release assay (Biddison, W.E. 1991, Current

20 Protocols in Immunology, p7,17.1-7.17.5, Ed. J. Coligan et al., J. Wiley and Sons, New York), using target cells that express the appropriate HLA molecule, in the presence and absence of the immunogenic peptide. Viability is determined by the exclusion of trypan blue dye by live cells. Cells are

25 tested for the presence of endotoxin by conventional techniques. Finally, the presence of bacterial or fungal contamination is determined by appropriate microbiological methods (chocolate agar, etc.). Once the cells pass all quality control and safety tests, they are washed and placed

30 in the appropriate infusion solution (Ringer/glucose lactate) and infused intravenously into the patient.

Example 10

Assays for CTL Activity

35 1. Peptide synthesis: Peptide syntheses were carried out by sequential coupling of N- α -Fmoc-protected amino acids on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer using standard Fmoc coupling cycles (software

version 1.40). All amino acids, reagents, and resins were obtained from Applied Biosystems or Bachem. Solvents were obtained from Burdick & Jackson. Solid-phase synthesis was started from an appropriately substituted Fmoc-amino acid-Sasrin resin. The loading of the starting resin was 0.5-0.7 mmol/g polystyrene, and 0.1 or 0.25 meq were used in each synthesis. A typical reaction cycle proceeded as follows: 1) The N-terminal Fmoc group was removed with 25% piperidine in dimethylformamide (DMF) for 5 minutes, followed by another treatment with 25% piperidine in DMF for 15 minutes. The resin was washed 5 times with DMF. An N-methylpyrrolidone (NMP) solution of a 4 to 10 fold excess of a pre-formed 1-hydroxybenzotriazole ester of the appropriate Fmoc-amino acid was added to the resin and the mixture was allowed to react for 30-90 min. The resin was washed with DMF in preparation for the next elongation cycle. The fully protected, resin bound peptide was subjected to a piperidine cycle to remove the terminal Fmoc group. The product was washed with dichloromethane and dried. The resin was then treated with trifluoroacetic acid in the presence of appropriate scavengers [e.g. 5% (v/v) water] for 60 minutes at 20°C. After evaporation of excess trifluoroacetic acid, the crude peptide was washed with dimethyl ether, dissolved in water and lyophilized. The peptides were purified to >95% homogeneity by reverse-phase HPLC using H₂O/CH₃CN gradients containing 0.2% TFA modifier on a Vydac, 300Å pore-size, C-18 preparative column. The purity of the synthetic peptides was assayed on an analytical reverse-phase column, and their composition ascertained by amino acid analysis and/or sequencing. Peptides were routinely dissolved in DMSO at the concentration of 20 mg/ml.

2. Media: RPMI-1640 containing 10% fetal calf serum (FCS) 2 mM Glutamine, 50 µg/ml Gentamicin and 5x10⁻⁵M 2-mercaptoethanol served as culture medium and will be referred to as R10 medium.

RPMI-1640 containing 25 mM Hepes buffer and supplemented with 2% FCS was used as cell washing medium.

3. Rat Concanavalin A supernatant: The spleen cells obtained from Lewis rats (Sprague-Dawley) were resuspended at a concentration of 5×10^6 cells/ml in R10 medium supplemented with 5 μ g/ml of ConA in 75 cm² tissue culture flasks. After 48 hr at 37°C, the supernatants were collected, supplemented with 1% α -methyl-D-mannoside and filter sterilized (.45 μ m filter). Aliquots were stored frozen at -20°C.

4. LPS-activated lymphoblasts: Murine splenocytes were resuspended at a concentration of $1-1.5 \times 10^6$ /ml in R10 medium supplemented with 25 μ g/ml LPS and 7 μ g/ml dextran sulfate in 75 cm² tissue culture flasks. After 72 hours at 37°C, the lymphoblasts were collected for use by centrifugation.

5. Peptide coating of lymphoblasts: Coating of the LPS activated lymphoblasts was achieved by incubating 30×10^6 lymphoblasts with 100 μ g of peptide in 1 ml of R10 medium for 1 hr at 37°C. Cells were then washed once and resuspended in R10 medium at the desired concentration for use in in vitro CTL activation.

6. Peptide coating of Jurkat A2/K^b cells: Peptide coating was achieved by incubating 10×10^6 irradiated (20,000 rads) Jurkat A2.1/K^b cells with 20 μ g of peptide in 1 ml of R10 medium for 1 hour at 37°C. Cells were washed three times and resuspended at the required concentration in R10 medium.

7. In Vitro CTL activation: One to four weeks after priming spleen cells (5×10^6 cells/well or 30×10^6 cells/T25 flask) were concultured at 37°C with syngeneic, irradiated (3,000 rads), peptide coated lymphoblasts (2×10^6 cells/well or 10×10^6 cells/T25 flask) in R10 medium to give a final volume of 2 ml in 24-well plates or 10 ml in T25 flasks.

8. Restimulation of effector cells: Seven to ten days after the initial in vitro activation, described in paragraph 7 above, a portion of the effector cells were restimulated with irradiated (20,000 rads), peptide-coated Jurkat A2/K^b cells (0.2×10^6 cells/well) in the presence of 3×10^6 "feeder cells"/well (C57Bl/6 irradiated spleen cells) in R10 medium supplemented with 5% rat ConA supernatant to help provide all of the cytokines needed for optimal effector cell growth.

9. Assay for cytotoxic activity: Target cells (3×10^6) were incubated at 37°C in the presence of $200\ \mu\text{l}$ of sodium ^{51}Cr chromate. After 60 minutes, cells were washed three times and resuspended in R10 medium. Peptides were added at the required concentration. For the assay, 10^4 ^{51}Cr -labeled target cells were added to different concentrations of effector cells (final volume of $200\ \mu\text{l}$) in U-bottom 96-2311 plates. After a 6-hour incubation period at 37°C , $0.1\ \text{ml}$ aliquots of supernatant were removed from each well and radioactivity was determined in a Micromedic automatic gamma counter. The percent specific lysis was determined by the formula: percent specific release = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Where peptide titrations were performed, the antigenicity of a given peptide (for comparison purposes) was expressed as the peptide concentration required to induce 40% specific ^{51}Cr release at a given E:T.

Transgenic mice were injected subcutaneously in the base of the tail with an incomplete Freund's adjuvant emulsion containing $50\ \text{nM}$ of the putative CTL epitopes containing the A2.1 motifs, and $50\ \text{nM}$ of a hepatitis B core T helper epitope. Eight to 20 days later, animals were sacrificed and spleen cells were restimulated in vitro with syngeneic LPS lymphoblasts coated with the putative CTL epitope. A source of IL-2 (rat con A supernatant) was added at day 6 of the assay to a final concentration of 5% and CTL activity was measured on day 7. The capacity of these effector T cells to lyse peptide-coated target cells that express the A2 KB molecule (Jurkat A2 KB) was measured as lytic units. The results are presented in Table 24.

The results of this experiment indicate that those peptides having a binding of at least 0.01 are capable of inducing CTL. All of the peptides in Appendices 1 and 2 having a binding of at least about 0.01 would be immunogenic.

TABLE 24
Binding and Immunogenicity
HBV Polymerase (ayw)

Peptide	Binding**	CTL Activity	Algorithm
1 2 3 4 5 6 7 8 9			
F L L S L G I H L	0.52	63	-20.8
G L Y S S T V P V	0.15	10	-21.9
H L Y S H P I I L	0.13	10	-21.1
W I L R G T S F V	0.018	-+	-20.9
N L S W L S L D V	0.013	6	-24.7
L L S S N L S W L	0.005	-	-21.7
N L Q S L T N L L	0.003	-	-23.9
H L L V G S S G L	0.002	-	-24.7
L L D D E A G P L	0.0002	-	-25.5
P L E E E L P R L	0.0001	-	-26.1
D L N L G N L N V	-*	-	-25.7
N L Y V S L L L L	-	-	-23.6
P L P I H T A E L	-	-	-25.04

*-=<0.0001

** Relative binding capacity compared to std with $IC_{50} = 52mM$
xxx Lytic units/ 10^6 cells; 1 lytic unit = the number of
effector cells required to give 30% Cr^{51} release.

-, -+ no measurable cytotoxic activity.

Example 11Identification of immunogenic peptides

Using the motifs identified above for HLA-A2.1 allele amino acid sequences from a tumor-related protein, Melanoma Antigen-1 (MAGE-1), were analyzed for the presence of these motifs. Sequences for the target antigen are obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs is done using the "FINDPATTERNS" program (Devereux et al., Nucleic Acids Research 12:387-395 (1984)).

Other viral and tumor-related proteins can also be analyzed for the presence of these motifs. The amino acid sequence or the nucleotide sequence encoding products is obtained from the GenBank database in the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu).

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs are identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr are added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 is derived from 9 viral isolates. Motifs are identified on those regions that have no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates are identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral isolates (10-12 viruses) was obtained from the Los Alamos National Laboratory database (May 1991 release) and analyzed in order to identify motifs that are constant throughout most viral isolates. Motifs that bear a

small degree of variation (one residue, in 2 forms) were also added to the peptide list.

Appendices 1 and 2 provide the results of searches of the following antigens cERB2, EBNA1, HBV, HCV, HIV, HPV, MAGE, p53, and PSA. Only peptides with binding affinity of at least 1% as compared to the standard peptide in assays described in Example 5 are presented. Binding as compared to the standard peptide is shown in the far right column. The column labeled "Pos." indicates the position in the antigenic protein at which the sequence occurs.

Example 12

Identification of immunogenic peptides

Using the motifs disclosed here, amino acid sequences from various antigens were screened for further motifs. Screening was carried out as described in Example 11. Tables 25 and 26 provide the results of searches of the following antigens cERB2, CMV, Influenza A, HBV, HIV, HPV, MAGE, p53, PSA, Hu S3 ribosomal protein, LCMV, and PAP. Only peptides with binding affinity of at least 1% as compared to the standard peptide in assays described in Example 5 are presented. Binding as compared to the standard peptide is shown for each peptide.

TABLE 25

Sequence	Antigen	Molecule	A2 Bind.
KIFGSLAFL	c-ERB2		0.1500
RILHNGAYSL	c-ERB2		0.0180
IISAVVGILL	c-ERB2		0.0120
MMWFVVLTV	CMV		0.7600
YLLLYFSPV	CMV		0.7500
YLYRLNFCL	CMV		0.7200
FMWTYLVTL	CMV		0.6800
LLWWITILL	CMV		0.4900
GLWCVLFFV	CMV		0.4700
LMIRGVLEV	CMV		0.4500
LLLCRLPFL	CMV		0.4200
AMSRNLFRV	CMV		0.1500
AMLTACVEV	CMV		0.1000
RLQPNVPLV	CMV		0.0480
VLARTFTPV	CMV		0.0440
RLLRGLIRL	CMV		0.0370
WMWFPSVLL	CMV		0.0360
YLCCGITLL	CMV		0.0210
SLLTEVETV	FLU-A	M1	0.0650
LLTEVETV	FLU-A	M1	0.2000
LLTEVETVVL	FLU-A	M1	0.0130
GILGFVFTL	FLU-A	M1	0.1900
GILGFVFTLT	FLU-A	M1	0.0150
ILGFVFTLT	FLU-A	M1	0.2600
ILGFVFTLTV	FLU-A	M1	0.0550
ALASCMGLI	FLU-A	M1	0.0110
RMGAVTTEV	FLU-A	M1	0.0200

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
VTTEVAFGL	FLU-A	M1	0.0360
MVTTTNPLI	FLU-A	M1	0.0150
FTFSPTYKA	HBV	POL	0.0190
YLHTLWKAGI	HBV	POL	0.0280
LMLQAGFFLV	HBV (a)	ENV (a)	0.6300
RMLTIPQSV	HBV (a)	ENV (a)	0.0580
SLDSWWTSTV	HBV (a)	ENV (a)	0.1000
FMLLLCLIFL	HBV (a)	ENV (a)	0.0450
LLPFVQWFV	HBV (a)	ENV (a)	0.6500
LMPFVQWFV	HBV (a)	ENV (a)	0.8300
FLGLSPTVWV	HBV (a)	ENV (a)	0.0300
SMLSPFLPLV	HBV (a)	ENV (a)	0.9700
GLWIRTPPV	HBV (a)	ENV (a)	0.3600
NLGNLNVSV	HBV (a)	ENV (a)	0.0160
YLHTLWKAGV	HBV (a)	POL (a)	0.1500
RLTGGVFLV	HBV (a)	POL (a)	0.1600
RMTGGVFLV	HBV (a)	POL (a)	0.1500
RLTGGVFLV	HBV (a)	ENV (a)	0.1600
ILGLLGFAV	HBV (a)	ENV (a)	0.0600
GLCQVFADV	HBV (a)	ENV (a)	0.0300
WLLRGTSFV	HBV (a)	ENV (a)	0.1000
YLPSALNPV	HBV (a)	ENV (a)	0.3200
LLVPFVQWFA	HBV adr		0.2600
FLPSDFFPSI	HBV adr		0.2100
VVSYVNVNM	HBV adr		0.0100
HLPDRVHFA	HBV adr		0.0160
SLAFSAVPA	HBV adr		0.0340

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
FLLTKILTI	HBV adw		0.6300
SLYNILSPFM	HBV adw		0.0440
CLFHIVNLI	HBV adw		0.2100
RLPDRVHFA	HBV adw		0.0940
ALPPASPSA	HBV adw		0.0710
GLLGWSPQA	HBV ayw		0.8650
FLGPLLVLQA	HBV ayw		0.0190
FLLTRILTI	HBV ayw		0.9300
GMLPVCPLI	HBV ayw		0.0520
QLFHLCLII	HBV ayw		0.0390
KLCIGWLWGM	HBV ayw		0.0210
LLWFHISCLI	HBV ayw		0.0130
YLVSFGVWI	HBV ayw		2.7000
LLEDWGPCA	HBV ayw		0.0180
KLHLYSHPI	HBV ayw		0.2900
FLLAQFTSA	HBV ayw		0.6600
LLAQFTSAI	HBV ayw		9.6000
YMDDVVLGA	HBV ayw		0.1600
ALMPYACI	HBV ayw		0.2000
GLCQVFADA	HBV ayw		0.0180
HLPDLVHFA	HBV ayw		0.1100
RLCCQLDPA	HBV ayw		0.0290
ALMPYACI	HBV ayw polymerase		0.5000
FLCKQYLNL	HBV ayw polymerase 665-673		0.0210

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
SLYADSPSV	HBV polymerase		0.3500
ALMPYASI	HBV polymerase		0.0760
NLNNLNVSI	HBV polymerase		0.0660
ALSLIVNLL	HBV polymerase		0.0470
KLHLYSHPI	HBV polymerase		0.2900
WILRGTSFV	HBV polymerase 1344-1352		0.0270
LVLQAGFFLL	HBVadr	ENV	0.0150
FILLCLIFL	HBVadr	ENV	0.0280
WILRGTSFV	HBVadr	POL	0.0180
IISCTCPTV	HBVadw	PreCore	0.0190
LVPFVQWFV	HBVadw	ENV	0.0200
LIISCSCPTV	HBVadw	CORE	0.0290
FLPSDFFPSI	HBVayr	PreCore	0.2100
LLCLGWLWGM	HBVayr	PreCore	0.0220
QLFHLCLII	HBVayw	PreCore	0.0390
CLGWLTGMDI	HBVayw	PreCore	0.0190
FLGGTTVCL	HBVayw	ENV	0.1700
SLYSILSPFL	HBVayw	ENV	0.2000
FLPSDFFPSV	HBVayw	CORE	1.5000
ILCWGELMTL	HBVayw	CORE	0.1900
LMTLATWVG	HBVayw	CORE	0.6800
TLATWVGVL	HBVayw	CORE	0.5700

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
GLSRYVARL	HBVayw	POL	0.1200
FLCKQYLNL	HBVayw	POL	0.1700
RMRGTFSAPL	HBVayw	POL	0.0110
SLYADSPSV	HBVayw	POL	0.3500
YLYGVGSAV	HCV		0.1600
LLSTTEWQV	HCV		0.0480
IIGAETFYV	HIV	POL	0.0260
QLWVTVYYGV	HIV	ENV	0.0250
NLWVTVYYGV	HIV	ENV	0.0160
KLWVTVYYGV	HIV	ENV	0.0150
KLWVTVYYGV	HIV.MN gp160		0.0150
YMLDLQPET	HPV16	E7	1.4000
TLGIVCPI	HPV16	E7	0.6500
YLLDLQEPV	HPV16 (a)	E7 (a)	0.2200
YMLDLQPEV	HPV16 (a)	E7 (a)	1.9000
MLDLQPETT	HPV16E7	E7	0.0130
SLQDIEITCVYCKTV	HPV18	E6	0.0100
RLTSLFFL	HSV		0.3400
RLTSLFFL	HSV		0.3400
LLLYDYDSL	HSV		0.2800
DMLGRVFFV	HSV		0.0110
TMFEALPHI	LCMV	Gp	0.2000
ALISFLLLA	LCMV	Gp	0.2200
TLMSIVSSL	LCMV	Gp	0.2000
NISGYNFSL	LCMV	Np	0.0280
ALLDGGNML	LCMV	Np	0.0320

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
ALHLFKITTV	LCMV	Gp	0.0170
SLISDQLLM	LCMV	Gp	0.0540
WLVNNGSYL	LCMV	Gp	0.0180
ALMDLLMFS	LCMV	Gp	0.4300
IMDLLMFST	LCMV	Gp	0.0460
LMFSTSAYL	LCMV	Gp	0.3600
YLVSIPLHL	LCMV	Gp	0.4200
SLHCKPEEA	MAGE1		0.0130
ALGLVCVQA	MAGE1		0.0150
LVLGTLLEV	MAGE1		0.0320
GTLEEVPTA	MAGE1		0.0130
CILESLFRA	MAGE1		0.0460
KVADLVGFLL	MAGE1		0.0560
KVADLVGFLLL	MAGE1		0.0200
VMIAEGGHA	MAGE1		0.0360
SMHCKPEEV	MAGE1 (a)		0.0180
AMGLVCVQV	MAGE1 (a)		0.0120
LMLGTLLEV	MAGE1 (a)		0.1300
KMADLVGFLV	MAGE1 (a)		1.5000
VMVTCLGLSV	MAGE1 (a)		0.3000
LLGDNQIMV	MAGE1 (a)		0.0430
QMMPKTGFLV	MAGE1 (a)		0.0500
VMIAEGGHV	MAGE1 (a)		0.0530
WMELSVMEV	MAGE1 (a)		0.0410
FLWGPRALA	MAGE1N		0.0420
RALAETSYV	MAGE1N		0.0100
ALAETSYVKVL	MAGE1N		0.0120

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
ALAETSYVKV	MAGE1N		0.0150
KVLEYVIKV	MAGE1N		0.0900
YVIKVSARV	MAGE1N		0.0140
ALREEEGV	MAGE1N		0.0210
YMFLWGPRV	MAGE1N (a)		0.2200
KMVELVHFLLL	MAGE2		0.6700
KMVELVHFL	MAGE2		0.1600
KMVELVHFLL	MAGE2		0.1100
KASEYLQLV	MAGE2		0.0110
YLQLVFGIEV	MAGE2		0.3700
LVFGIEVVEV	MAGE2		0.0120
QLVFGIELMEV	MAGE3		0.3400
KVAELVHFL	MAGE3		0.0550
KVAELVHFLL	MAGE3		0.0120
ELMEVDPIGHL	MAGE3		0.0260
HLYIFATCLGL	MAGE3		0.0410
IMPKAGLLIIV	MAGE3		0.0130
LVFGIELMEV	MAGE3		0.1100
ALGRNSFEV	p53 264-272 A8 (A1)		0.0570
LLGANSFEV	p53 264-272 A8 (A4)		0.1100
LLGRASFEV	p53 264-272 A8 (A5)		0.2200
LLGRNAFEV	p53 264-272 A8 (A6)		0.0390
LLGRNSFAV	p53 264-272 A8 (A8)		0.0420
RLGRNSFEV	p53 264-272 A8 (R1)		0.0190
LLGRRSFEV	p53 264-272 A8 (R5)		0.0540
LLGRNSFRV	p53 264-272 A8 (R8)		0.0250
LLFFWLDRSV	PAP		0.6000

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
VLAKELKFV	PAP		0.0590
ILLWQPIPV	PAP		1.3000
IMYSAHDTTV	PAP		0.0610
FLTLSVTWI	PSA		0.0150
FLTLSVTWIGA	PSA		0.0160
FLTLSVTWI	PSA		0.0150
VLVHPQWVLT	PSA		0.0130
SLFHPEDTGQV	PSA		0.0190
MLLRLSEPAEL	PSA		0.1400
ALGTTCYA	PSA		0.0230
KLQCVDLHVI	PSA		0.0370
FLPSDYFPSV	HBVc18-27 analog		1.0000
YSFLPSDFFPSV	HBVc18-27 analog		0.0190

Table 26

Sequence	Antigen	Molecule	A2 Bind.
ALFLGFLGAA	HIV	gp160	0.4950
MLQLTVWGI	HIV	gp160	0.2450
RVIEVLQRA	HIV	gp160	0.1963
KLTPLCVTL	HIV	gp160	0.1600
LLIAARIVEL	HIV	gp160	0.1550
SLLNATDIAV	HIV	gp160	0.1050
ALFLGFLGA	HIV	gp160	0.0945
HMLQLTVWGI	HIV	gp160	0.0677
LLNATDIAV	HIV	gp160	0.0607
ALLYKLDIV	HIV	gp160	0.0362
WLWYIKIFI	HIV	gp160	0.0355
TIIVHLNESV	HIV	gp160	0.0350
LLQYWSQEL	HIV	gp160	0.0265
IMIVGGLVGL	HIV	gp160	0.0252
LLYKLDIVSI	HIV	gp160	0.0245
FLAIIWVDL	HIV	gp160	0.0233

Table 26 (Cont'd)

TLQCKIKQII	HIV	gp160	0.0200
GLVGLRIVFA	HIV	gp160	0.0195
FLGAAGSTM	HIV	gp160	0.0190
IISLWDQSL	HIV	gp160	0.0179
TVWGIKQLQA	HIV	gp160	0.0150
LLGRRGWEV	HIV	gp160	0.0142
AVLSIVNRV	HIV	gp160	0.0132
FIMIVGGLV	HIV	gp160	0.0131
LLNATDIAVA	HIV	gp160	0.0117
FLYGALLLA	PLP		1.9000
SLLTFMIAA	PLP		0.5300
FMIAATYNFAV	PLP		0.4950
RMYGVLPMI	PLP		0.1650
IAATYNFAV	PLP		0.0540
GLLECCARCLV	PLP		0.0515
YALTVVWLL	PLP		0.0415
ALTVVWLLV	PLP		0.0390
FLYGALLL	PLP		0.0345
SLCADARMYGV	PLP		0.0140
LLVFACSAV	PLP		0.0107

Table 26 (Cont'd)

Sequence	Antigen	A2
KMVELVHFL	MAGE2	0.2200
KVAELVHFL	MAGE3	0.0550
RALAETSYV	MAGE1N	0.0100
LVFGIELMEV	MAGE3	0.1100
FLWGPRALA	MAGE1N	0.0420
ALAETSYVKV	MAGE1	0.0150
LVLGTLEEV	HIV	0.0320
LLWKGEHAVV	HIV	0.0360
IIGAETFYV	HIV	0.0260
IMVTVYYGV	HIV	0.4400
LLFNILGGWV	HCV	3.5000
LLALLSCLTV	HCV	0.6100
YLVAYQATV	HCV	0.2500
FILLADARV	HCV	0.2300
ILAGYGAGV	HCV	0.2200
YLLPRRGPR	HCV	0.0730
GLGCIITSL	HCV	0.0610
DIMGYIPLV	HCV	0.0550
LLALLSCLTI	HCV	0.0340
VLAALAAAYCL	HCV	0.0110
LLVPFVQWFV	HBV	1.6000
FLLAQFTSA	HBV	0.6600
FLLSLGIHL	HBV	0.5200
AIMPLYACI	HBV	0.5000
ILLCLIFLL	HBV	0.3000
LLPIFFCLWV	HBV	0.1000
YLHTLWKAGI	HBV	0.0560

Table 26 (Cont'd)

YLHTLWKAGV	HBV	0.1300
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Example 13Identification of immunogenic peptides
in autoantigens

As noted above, the motifs of the present invention
5 can also be screened in antigens associated with autoimmune
diseases. Using the motifs identified above for HLA-A2.1
allele amino acid sequences from myelin proteolipid (PLP),
myelin basic protein (MBP), glutamic acid decarboxylase (GAD),
and human collagen types II and IV were analyzed for the
10 presence of these motifs. Sequences for the antigens were
obtained from Trifilieff et al., *C.R. Seances Acad. Sci.*
300:241 (1985); Eyler et al., *J. Biol. Chem.* 246:5770 (1971);
Yamashita et al. *Biochim. Biophys. Res. Comm.* 192:1347
(1993); Su et al., *Nucleic Acids Res.* 17:9473 (1989) and
15 Pihlajaniemi et al. *Proc. Natl. Acad. Sci. USA* 84:940 (1987).
The identification of motifs was done using the approach
described in Example 5 and the algorithms of Examples 6 and 7.
Table 27 provides the results of the search of these antigens.

Using the quantitative binding assays of Example 4,
20 the peptides are next tested for the ability to bind MHC
molecules. The ability of the peptides to suppress
proliferative responses in autoreactive T cells is carried out
using standard assays for T cell proliferation. For instance,
methods as described by Miller et al. *Proc. Natl. Acad. Sci.*
25 *USA*, 89:421 (1992) are suitable.

For further study, animal models of autoimmune
disease can be used to demonstrate the efficacy of peptides of
the invention. For instance, in HLA transgenic mice,
autoimmune model diseases can be induced by injection of MBP,
30 PLP or spinal cord homogenate (for MS), collagen (for
arthritis). In addition, some mice become spontaneously
affected by autoimmune disease (e.g., NOD mice in diabetes).
Peptides of the invention are injected into the appropriate
animals, to identify preferred peptides.

TABLE 27

Human PLP peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
3	9	L	L	E	C	C	A	R	C	L		A2.1	(LM)2; (LVI)c
23	9	G	L	C	F	F	G	V	A	L			
39	9	A	L	T	G	T	E	K	L	I			
134	9	S	L	E	R	V	C	H	C	L			
145	9	W	L	G	H	P	D	K	F	V			
158	9	A	L	T	V	V	W	L	L	V			
164	9	L	L	V	F	A	C	S	A	V			
205	9	R	M	Y	G	V	L	P	W	I			
2	10	G	L	L	E	C	C	A	R	C	L		
3	10	L	L	E	C	C	A	R	C	L	V		
10	10	C	L	V	G	A	P	F	A	S	L		
163	10	W	L	L	V	F	A	C	S	A	V		
250	10	T	L	V	S	L	L	T	F	M	I		
64	9	V	I	H	A	F	Q	Y	V	I			Algorithm
80	9	F	L	Y	G	A	L	L	L	A			
157	9	Y	A	L	T	V	V	W	L	L			
163	9	W	L	L	V	F	A	C	S	A			
234	9	Q	M	T	F	H	L	F	I	A			
251	9	L	V	S	L	L	T	F	M	I			
253	9	S	L	L	T	F	M	I	A	A			
259	9	I	A	A	T	Y	N	F	A	V			
84	10	A	L	L	L	A	E	G	F	Y	T		
157	10	Y	A	L	T	V	V	W	L	L	V		
165	10	L	V	F	A	C	S	A	V	P	V		
218	10	K	V	C	G	S	N	L	L	S	I		
253	10	S	L	L	T	F	M	I	A	A	T		

Table 27 continued

Human Collagen TypeIV peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
5	9	A	L	M	G	P	L	G	L	L		A2.1	(LM)2; (LVI)c
11	9	G	L	L	G	Q	I	G	P	L			
23	9	G	M	L	G	Q	K	G	E	I			
231	9	P	L	G	Q	D	G	L	P	V			
3	10	T	L	A	L	M	G	P	L	G	L		
24	10	M	L	G	Q	K	G	E	I	G	L		
59	10	P	L	G	K	D	G	P	P	G	V		
139	10	P	L	G	L	P	G	A	S	G	L		

Human Collagen TypeII peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
794	9	G	L	A	G	Q	R	G	I	V		A2.1	(LM)2; (LVI)c
17	9	V	M	Q	G	P	M	G	P	M			Algorithm

Table 27 continued

Human GAD peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
56	9	S	L	E	E	K	S	R	L	V		A2.1	(LM)2; (LVI)c
116	9	F	L	L	E	V	V	D	I	L			
117	9	L	L	E	V	V	D	I	L	L			
150	9	G	M	E	G	F	N	L	E	L			
157	9	E	L	S	D	H	P	E	S	L			
168	9	I	L	V	D	C	R	D	T	L			
190	9	Q	L	S	T	G	L	D	I	I			
229	9	T	L	K	K	M	R	E	I	V			
275	9	G	M	A	A	V	P	K	L	V			
300	9	A	L	G	F	G	T	D	N	V		A2.1	(LM)2; (LVI)c
409	9	V	L	L	Q	C	S	A	I	L			
410	9	L	L	Q	C	S	A	I	L	V			
416	9	I	L	V	K	E	K	G	I	L			
466	9	L	M	W	K	A	K	G	T	V			
534	9	K	L	H	K	V	A	P	K	I			
546	9	M	M	E	S	G	T	T	M	V			
582	9	F	L	I	E	E	I	E	R	L			
42	10	K	L	G	L	K	I	C	G	F	L		
116	10	F	L	L	E	V	V	D	I	L	L		
138	10	V	L	D	F	H	H	P	H	Q	L		
147	10	L	L	E	G	M	E	G	F	N	L		
212	10	N	M	F	T	Y	E	I	A	P	V		
275	10	G	M	A	A	V	P	K	L	V	L		
300	10	A	L	G	F	G	T	D	N	V	I		
328	10	I	L	E	A	K	Q	K	G	Y	V		
381	10	L	M	S	R	K	H	R	H	K	L		
409	10	V	L	L	Q	C	S	A	I	L	V		
435	10	L	L	Q	P	D	K	Q	Y	D	V		
465	10	W	L	M	W	K	A	K	G	T	V		
485	10	E	L	A	E	Y	L	Y	A	K	I		
545	10	L	M	M	E	S	G	T	T	M	V		
252	9	G	A	I	S	N	M	Y	S	I			
367	9	N	L	W	L	H	V	D	A	A			
567	9	R	M	V	I	S	N	P	A	A			
299	10	A	A	L	G	F	G	T	D	N	V		
406	10	M	M	G	V	L	L	Q	C	S	A		
423	10	I	L	Q	G	C	N	Q	M	C	A		

Algorithm

Example 14

Immunogenicity of HPV peptides in A2.1 transgenic mice

A group of 14 HPV peptides, including 9 potential epitopes plus 3 low binding and one non-binding peptides as controls was screened for immunogenicity in HLA-A2.1 transgenic mice using the methods described in Example 10. To test the immunogenic potential of the peptides, HLA A2.1 transgenic mice were injected with 50 μ g/mouse of each HPV peptide together with 140 μ g/mouse of helper peptide (HBV core 128-140 (TPPAYRPPNAPIL)). The peptides were injected in the base of the tail in a 1:1 emulsion IFA. Three mice per group were used. As a positive control, the HBV polymerase 561-570 peptide, which induced a strong CTL response in previous experiments, was utilized.

Based on these results (Table 28), four unrelated peptides were considered to be the most immunogenic: TLGIVCPI, LLMGTLGIV, YMLDLQPETT, and TIHDIILECV. TLGIVCPI and YMLDLQPETT were found to be good HLA-A2.1 binders, while LLMGTLGIV and TIHDIILECV were found to be intermediate binders in previous binding assays.

TABLE 28

HPV-16 Peptides for possible use in clinical trial

Peptide Position/ Cytel ID	Sequence	AA	A2.1 binding	Immunogenicity Experiment 1	Immunogenicity Experiment 2
E7.86/1088.01	TLGIVCPI	8	0.15	94.4 (1.34)	54.2 (1.43) *
E7.86/1088.06	TLGIVCPIC	9	0.075	2.05 (4.93)	1.3 (3.74)
E7.85/1088.08	GTLGIVCPI	9	0.021	9/08 (3.93)	-**
E7.11/1088.03	YMLDLQPETT	10	0.15	10.32 (1.66)	5.7 (2.39)
E7.11/1088.04	YMLDLQPET	9	0.14	5.0 (3.70)	2.6 (15.5)
E7.12/1088.09	MLDLQPETT	9	0.0028	-	-
E6.52/1088.05	FAFRDLCIV	9	0.057	-	ND
E7.82/1088.02	LLMGTLGIV	9	0.024	9.62 (2.53)	8.93 (1.91)
E6.29/1088.10	TIHDIILECV	10	0.021	22.13 (3.71)	0.4 (3.52)
E7.7/1088.07	TLHEYMLDL	9	0.0070	-	1.2 (3.88)
E6.18/1088.15	KLPQLCTEL	9	0.0009	-	0.3 (5.64)
E6.7/1088.11	AMFQDPQER	10	0.0002	-	ND
E6.26/1088.12	LQTTIHDI	9	0.0002	-	-
E7.73/1088.13	HVDIRTLED	9	0	-	ND

* Δ Lytic Units, geometric mean x+ SD (3 mice/peptide)

** a dash indicates Δ Lytic Units with a geometric mean ≤0.2

Mixtures of selected HPV epitopes

A combination of CTL peptides and a helper peptide were tested for the ability to provide an increased immune response. The four single peptides were injected separately in order to compare their immunogenicity to injections containing only the two good binders or only the two intermediate binders. In addition all four peptide were injected together. To further evaluate the immunogenicity of a combination of peptides with different binding affinity decreases, another control was introduced in this experiment. A mixture of the two good binders was injected in a different site than the mixture of the two intermediate binders into the base of the tail of the same mouse. All groups of CTL epitopes were injected together with the HBVc helper epitope, with the exception of two groups in which all four HPV coinjected with two different doses of a PADRE helper peptide (aKXVAAWTLKAAa, where a is d-alanine and X is cyclohexylalanine) either 1 μ g or 0.05 μ g per mouse.

All four peptides induced a strong CTL response when injected alone and tested using target cells labeled with the appropriate peptide (Table 29). TLGIVCPI proved to be the strongest epitope, an observation confirming the results described above. When mixtures of all four peptides were injected and the responses were stimulated in vitro and tested with target cells pulsed with each single peptide, all combinations showed a strong CTL response. No significant difference was observed when the two helper epitopes were compared. This might in part be due to the fact that the highest dose of PADRE used in this experiment was 140-fold lower than the one for the HBV helper peptide.

Injection of mixtures of the two good binders together or the two intermediate binders resulted in a very low CTL response in both cases even though the single peptides were highly effective. These results, however, are due to a very low number of cell recovery after splenocyte culture of 6 days and are therefore regarded as preliminary.

TABLE 29

HPV Peptides single and in combinations

A

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Peptide/s injected	Peptides in restimulation and CTL assay			
	1088.01	1088.02	1088.03	1088.10
same as in vitro	116.1 (3.49)*	55.98 (2.49)	5.56 (1.75)	16.4 (1.49)
1088.01 + 1088.03 + 875.23	1.37 (16.56)		0 (0)	
1088.02 + 1088.10 + 875.23		1.11 (2.9)		1.62 (13.1)
1088.01/.03 + 1088.02/.10 + 875.23	19.5 (4.1)	4.68 (2.3)	1.13 (21.9)	1.17 (2.58)
1088.all + 875.23	107.9 (4.77)	13.52 (1.4)	2.58 (5.07)	102.3 (1.32)
1088.all + PADRE 1 µg	73.11 (4.48)	16.83 (2.54)	3.55 (2.9)	20.13 (1.05)
1088.all + PADRE 0.05 µg	37.15 (2.25)	26.79 (2.09)	6.5 (1.64)	4.45 (4.14)

* Δ Lytic Units 30% geometric mean (+x deviation)

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Peptides were dissolved in 50%DMSO/H₂O to reach a stock concentration of 20mg/ml and were further dissolved in sterile PBS. For subcutaneous injection in the base of the tail of A2.1 transgenic mice, the peptide solution was mixed 1:1 with IFA. The injected amount of HPV-CTL peptides was 50 µg/mouse coinjected with 140 µg/mouse of the HBVcore peptide 875.23 or the indicated dose of PADRE (3 mice/group). Spleens were removed on day 11 and splenocytes were restimulated in vitro with irradiated LPS-Blasts pulsed with the indicated HPV-CTL epitopes at 1µg/ml. After six days, the cytotoxic assay was performed using Jurkat JA2Kb cells (A) or MBB17 (B) as target cells labelled with 51Cr in the presence or absence of the appropriate HPV epitope peptides.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All
5 publications, patents, and patent applications cited herein are hereby incorporated by reference.

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0841	ILSPFLPLL	9	HBV	adr	ENV	371	2.9
1.0240	TLQDIVLHL	9	HPV	18	E7	7	0.76
1.0838	WLSLLVPFV	9	HBV	adr	ENV	335	0.72
1.0851	FLLSLGIHL	9	HBV	adr	POL	1147	0.52
1.0306	QLFEDNYAL	9	c-ERB2			106	0.46
1.0814	LMVTVYYGV	9	HIV		ENV	2182	0.44
1.0878	MMWFWGPSL	9	HBV	adw	ENV	360	0.41
1.0839	MMWYWGPSL	9	HBV	adr	ENV	360	0.41
1.0384	FLTKQYLNL	9	HBV	adw	POL	1279	0.29
1.0321	ILHNGAYSL	9	c-ERB2			435	0.21
1.0834	LLLCLIFLL	9	HBV	adr	ENV	250	0.19
1.0167	GLYSSTVPV	9	HBV	adr	POL	635	0.15
1.0849	HLYSHPIIL	9	HBV	adr	POL	1076	0.13
1.0275	RMPEAAPPV	9	p53			65	0.12
1.0854	LLMGTLGIV	9	HPV	16	E7	82	0.11
1.0880	ILSPFMPLL	9	HBV	adw	ENV	371	0.11
1.0127	YLVAYQATV	9	HCV		LORF	1585	0.11
1.0151	VLLDYQGML	9	HBV	adr	ENV	259	0.11
1.0018	VLAEMSQV	9	HIV		GAG	367	0.11
1.0330	RLLQETELV	9	c-ERB2			689	0.091
1.0209	SLYAVSPSV	9	HBV	adr	POL	1388	0.078
1.0816	DLMGYIPLV	9	HCV		CORE	132	0.055
1.0835	LLCLIFLLV	9	HBV	adr	ENV	251	0.049
1.0852	FLCQQYLHL	9	HBV	adr	POL	1250	0.048

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0882	NLYVSLMLL	9	HBV	adw	POL	1088	0.046
1.0837	GMLPVCPLL	9	HBV	adr	ENV	265	0.046
1.0819	ILPCSFTTL	9	HCV		NS1/ENV2	676	0.045
1.0109	ALSTGLIHL	9	HCV		NS1/ENV2	686	0.042
1.0833	ILLCLIFL	9	HBV	adr	ENV	249	0.035
1.0301	HLYQGCQVV	9	c-ERB2			48	0.034
1.0337	CLTSTVQLV	9	c-ERB2			789	0.034
1.0842	PLLPIFFCL	9	HBV	adr	ENV	377	0.031
1.0861	ALCRWGLLL	9	c-ERB2			5	0.031
1.0309	VLIQRNPQL	9	c-ERB2			153	0.029
1.0828	VLQAGFFLL	9	HBV	adr	ENV	177	0.024
1.0844	LLWFHISCL	9	HBV	adr	CORE	490	0.024
1.0135	ILAGYGAGV	9	HCV		LORF	1851	0.024
1.0870	QLMPYGCLL	9	c-ERB2			799	0.023
1.0075	LLWKGEHAV	9	HIV		POL	1496	0.023
1.0873	FLGGTPVCL	9	HBV	adw	ENV	204	0.021
1.0323	ALIHNNTHL	9	c-ERB2			466	0.021
1.0859	VLVHPQWVL	9	PSA			49	0.020
1.0267	KLQCVDLHV	9	PSA			166	0.019
1.0820	VLPCSFTTL	9	HCV		NS1/ENV2	676	0.017
1.0111	HLHQNIQDV	9	HCV		NS1/ENV2	693	0.016
1.0103	SMVGNWAKV	9	HCV		ENV1	364	0.016
1.0283	LLGRNSFEV	9	p53			264	0.014
1.0207	GLYRPLLSL	9	HBV	adr	POL	1370	0.014

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0389	GLYRPLLRL	9	HBV	adw	POL	1399	0.014
1.0185	NLSWLSLDV	9	HBV	adr	POL	996	0.013
1.0113	FLLLADARV	9	HCV		NS1/ENV2	725	0.013
1.0119	YLVTRHADV	9	HCV		LORF	1131	0.011
1.0846	CLTHIVNLL	9	HBV	adr	POL	912	0.010
1.0156	ELMNLATWV	9	HBV	adr	CORE	454	0.010
1.0236	KLPDLCTEL	9	HPV	18	E6	13	0.010
1.0056	ALQDSGLEV	9	HIV		POL	1180	0.0083
1.0375	LLSSDLSWL	9	HBV	adw	POL	1021	0.0081
1.0094	ALAHGVRVL	9	HCV		CORE	150	0.0072
1.0129	TLHGPTPLL	9	HCV		LORF	1617	0.0070
1.0041	KLLRGTKAL	9	HIV		POL	976	0.0069
1.0131	CMSADLEV	9	HCV		LORF	1648	0.0067
1.0872	GLLGPLLVL	9	HBV	adw	ENV	170	0.0066
1.0228	TLHEYMLDL	9	HPV	16	E7	7	0.0059
1.0274	KLLPENNV	9	p53			24	0.0058
1.0043	ILKEPVHGV	9	HIV		POL	1004	0.0055
1.0206	RLGLYRPLL	9	HBV	adr	POL	1368	0.0050
1.0188	GLPRYVARL	9	HBV	adr	POL	1027	0.0050
1.0202	KLIGTDNSV	9	HBV	adr	POL	1317	0.0050
1.0818	FLALLSCL	9	HCV		CORE	177	0.0046
1.0184	LLSSNLSWL	9	HBV	adr	POL	992	0.0046
1.0102	QLLRIPQAV	9	HCV		ENV1	337	0.0039
1.0114	GLRDLAVAV	9	HCV		LORF	963	0.0034

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0005	TLNAWVKVI	9	HIV		GAG	156	0.0032
1.0183	NLQSLTNLL	9	HBV	adr	POL	985	0.0025
1.0359	QLGRKPTPL	9	HBV	adw	ENV	89	0.0025
1.0150	SLDSWWTSL	9	HBV	adr	ENV	194	0.0023
1.0362	ILSKTGDVP	9	HBV	adw	ENV	153	0.0021
1.0866	ILLVVVLGV	9	c-ERB2			661	0.0020
1.0214	LLHKRTLGL	9	HBV	adr	"X"	1510	0.0019
1.0216	CLFKDWEEL	9	HBV	adr	"X"	1533	0.0019
1.0862	GLGISWLGL	9	c-ERB2			447	0.0018
1.0187	HLLVGSSGL	9	HBV	adr	POL	1020	0.0018
1.0318	TLEEITGYL	9	c-ERB2			402	0.0018
1.0328	PLTSIISAV	9	c-ERB2			650	0.0015
1.0822	LLGCIITSL	9	HCV		LORF	1039	0.0015
1.0277	ALNKMFCQL	9	p53			129	0.0013
1.0066	HLEGKIILV	9	HIV		POL	1322	0.0010
1.0308	QLRSLTEIL	9	c-ERB2			141	0.0008
1.0115	DLAVAVEPV	9	HCV		LORF	966	0.0008
1.0391	VLHKRTLGL	9	HBV	adw	"X"	1539	0.0007
1.0876	FLCILLCL	9	HBV	adw	ENV	246	0.0007
1.0148	LLDPRVRGL	9	HBV	adr	ENV	120	0.0006
1.0221	KLPQLCTEL	9	HPV	16	E6	18	0.0006
1.0065	HLEGKVILV	9	HIV		POL	1322	0.0006
1.0017	EMMTACQGV	9	HIV		GAG	350	0.0006
1.0055	HLALQDSGL	9	HIV		POL	1178	0.0005

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0868	VLGVVFGIL	9	c-ERB2			666	0.0005
1.0004	TLNAWVKVV	9	HIV		GAG	156	0.0005
1.0381	HLESLYAAV	9	HBV	adw	POL	1165	0.0005
1.0128	CLIRLKPTL	9	HCV		LORF	1610	0.0004
1.0255	CLGLSYDGL	9	MAGE	1/3		174	0.0004
1.0212	HLSLRGLPV	9	HBV	adr	"X"	1470	0.0004
1.0247	ILESIFRAV	9	MAGE	1		93	0.0004
1.0092	TLTCGFADL	9	HCV		CORE	125	0.0003
1.0108	TLPALSTGL	9	HCV		NS1/ENV2	683	0.0003
1.0294	ALAIPQCRL	9	EBNA1			525	0.0003
1.0101	DLCGSVFLV	9	HCV		ENV1	280	0.0003
1.0231	RLCVQSTHV	9	HPV	16	E7	66	0.0003
1.0162	LLDDEAGPL	9	HBV	adr	POL	587	0.0002
1.0829	CLRRFIIFL	9	HBV	adr	ENV	239	0.0002
1.0126	GLPVCQDHL	9	HCV		LORF	1547	0.0001
1.0163	PLEEELPRL	9	HBV	adr	POL	594	0.0001
1.0130	PLLYRLGAV	9	HCV		LORF	1623	0.0001
1.0042	ELAENREIL	9	HIV		POL	997	0
1.0054	ELQAIHLAL	9	HIV		POL	1173	0
1.0089	LIPRRGPRL	9	HCV		CORE	36	0
1.0091	NLGKVIDTL	9	HCV		CORE	118	0
1.0093	PLGGAARAL	9	HCV		CORE	143	0
1.0154	DLLDTASAL	9	HBV	adr	CORE	419	0
1.0178	QLKQSRLGL	9	HBV	adr	POL	791	0

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0179	GLQPQGGSL	9	HBV	adr	POL	798	0
1.0286	PLDGEYFTL	9	p53			322	0
1.0296	VLKDAIKDL	9	EBNA1			574	0
1.0310	QLCYQDTIL	9	c-ERB2			160	0
1.0007	DLNTMLNTV	9	HIV		GAG	188	0
1.0037	ELHPDKWTV	9	HIV		POL	928	0
1.0070	ELKKIIGQV	9	HIV		POL	1412	0
1.0157	ELVVSIVNV	9	HBV	adr	CORE	473	0
1.0160	CLTFGRETV	9	HBV	adr	CORE	497	0
1.0164	DLNLGNLNV	9	HBV	adr	POL	614	0
1.0867	LLVVVLGVV	9	c-ERB2			662	0
1.0159	NMGLKIRQL	9	HBV	adr	CORE	482	0
1.0322	SLRELGSGL	9	c-ERB2			457	<0.0002
1.0350	DLLEKGERL	9	c-ERB2			933	<0.0002
1.0352	DLVDAEAYL	9	c-ERB2			1016	<0.0002
1.0366	PLEEELPHL	9	HBV	adw	POL	623	<0.0002
1.0372	DLQHGRVL	9	HBV	adw	POL	781	<0.0002
1.0390	PLPGPLGAL	9	HBV	adw	"X"	1476	<0.0002
1.0811	LLTQIGCTL	9	HIV		POL	685	<0.0002
1.0812	PLVKLWYQL	9	HIV		POL	1116	<0.0002
1.0832	FLFILLCL	9	HBV	adr	ENV	246	<0.0002
1.0847	NLYVSLLL	9	HBV	adr	POL	1059	<0.0002
1.0316	PLQPEQLQV	9	c-ERB2			391	<0.0002
1.0342	DLAARNVLV	9	c-ERB2			845	<0.0002

APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0343	VLVKSPNHV	9	c-ERB2			851	<0.0002
1.0356	TLSPGKNGV	9	c-ERB2			1172	<0.0002
1.0376	DLSWLSLDV	9	HBV	adw	POL	1025	<0.0002
1.0363	NMENIASGL	9	HBV	adw	ENV	163	<0.0002
1.0195	TLPQEHIVL	9	HBV	adr	POL	1179	<0.0003
1.0196	KLKQCFRKL	9	HBV	adr	POL	1188	<0.0003
1.0201	PLPIHTAEL	9	HBV	adr	POL	1296	<0.0003
1.0210	QLDPARDVL	9	HBV	adr	"X"	1426	<0.0003
1.0220	VLGGCRHKL	9	HBV	adr	"X"	1551	<0.0003
1.0229	DLQPETTDL	9	HPV	16	E7	14	<0.0003
1.0245	ALEAQQEAL	9	MAGE	1		15	<0.0003
1.0266	DLPTQEPAL	9	PSA			136	<0.0003
1.0279	HLIRVEGNL	9	p53			193	<0.0003
1.0282	TLEDSSGNL	9	p53			256	<0.0003
1.0238	ELRHYSDSV	9	HPV	18	E6	77	<0.0003
1.0268	DLHVISNDV	9	PSA			171	<0.0003
1.0836	CLIFLLVLL	9	HBV	adr	ENV	253	<0.0006

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0890	LLFNILGGWV	10	HCV		LORF	1807	3.5
1.0930	LLVPFVQWFV	10	HBV	adw	ENV	338	1.6
1.0884	LLALLSCLTV	10	HCV		CORE	178	0.61
1.0896	ILLCLIFLL	10	HBV	adr	ENV	249	0.30
1.0518	GLSPTVWLSV	10	HBV	adr	ENV	348	0.28
1.0902	SLYNILSPFL	10	HBV	adr	ENV	367	0.23
1.0892	LLVLQAGFFL	10	HBV	adr	ENV	175	0.21
1.0686	FLQTHIFAEV	10	EBNA1			565	0.17
1.0628	QLFLNTLSFV	10	HPV	18	E7	88	0.11
1.0904	LLPIFFCLWV	10	HBV	adr	ENV	378	0.10
1.0897	LLLCLIFLLV	10	HBV	adr	ENV	250	0.099
1.0516	LLDYQGMLPV	10	HBV	adr	ENV	260	0.085
1.0901	WMMWYWGPSL	10	HBV	adr	ENV	359	0.084
1.0533	GLYSSTVPVL	10	HBV	adr	POL	635	0.080
1.0469	YLLPRRGPRL	10	HCV		CORE	35	0.073
1.0888	GLGCIITSL	10	HCV		LORF	1038	0.061
1.0907	ILCWGELMNL	10	HBV	adr	CORE	449	0.052
1.0927	LLGICLTSTV	10	c-ERB2			785	0.049
1.0452	LLWKGEHAVV	10	HIV		POL	1496	0.036
1.0885	LLALLSCLTI	10	HCV		CORE	178	0.034
1.0620	KLTNTGLYNL	10	HPV	18	E6	92	0.032
1.0502	RLIVFPDLGV	10	HCV		LORF	2578	0.032
1.0659	FLTPKKLQCV	10	PSA			161	0.031
1.0932	WMMWFVGPSL	10	HBV	adw	ENV	359	0.029

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0772	SLNFLGGTPV	10	HBV	adw	ENV	201	0.027
1.0609	SLQDIEITCV	10	HPV	18	E6	24	0.025
1.0526	ILSTLPETTV	10	HBV	adr	CORE	529	0.022
1.0508	RLHGLSAFSL	10	HCV		LORF	2885	0.020
1.0493	ILGGWVAAQL	10	HCV		LORF	1811	0.018
1.0738	VMAGVGSPYV	10	c-ERB2			773	0.018
1.0460	QLMVTVYYGV	10	HIV		ENV	2181	0.017
1.0573	ILRGTSFVYV	10	HBV	adr	POL	1345	0.016
1.0703	SLTEILKGGV	10	c-ERB2			144	0.015
1.0912	LLGCAANWIL	10	HBV	adr	POL	1337	0.014
1.0798	ALPPASPSAV	10	HBV	adw	"X"	1483	0.013
1.0908	QLLWFHISCL	10	HBV	adr	CORE	489	0.013
1.0677	NLLGRNSFEV	10	p53			263	0.013
1.0889	VLAALAAYCL	10	HCV		LORF	1666	0.011
1.0528	LLLDDEAGPL	10	HBV	adr	POL	586	0.011
1.0500	IMAKNEVFCV	10	HCV		LORF	2558	0.0088
1.0492	VLVGGVLAAL	10	HCV		LORF	1661	0.0084
1.0898	LLCLIFLLVL	10	HBV	adr	ENV	251	0.0075
1.0458	KLMVTVYYGV	10	HIV		ENV	2181	0.0069
1.0459	NLMVTVYYGV	10	HIV		ENV	2181	0.0067
1.0530	GLSPTVWLSA	10	HBV	adw	ENV	348	0.0067
1.0759	SLPTHDPSP	10	c-ERB2			1100	0.0059
1.0419	VLPEKDSWTV	10	HIV		POL	940	0.0056
1.0666	FLHSGTAKSV	10	p53			113	0.0050

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0473	GLIHLHQIV	10	HCV		NS1/ENV2	690	0.0047
1.0792	SLYAAVTNFL	10	HBV	adw	POL	1168	0.0046
1.0780	IMPARFYPNV	10	HBV	adw	POL	713	0.0043
1.0507	YLTRDPTTPL	10	HCV		LORF	2803	0.0042
1.0914	GLYNLLIRCL	10	HPV	18	E6	97	0.0036
1.0649	YLEYGRCRTV	10	MAGE	1		248	0.0034
1.0561	SLFTSITNFL	10	HBV	adr	POL	1139	0.0034
1.0788	NLLSSDLSWL	10	HBV	adw	POL	1020	0.0032
1.0753	RMARDPQRFV	10	c-ERB2			978	0.0020
1.0568	RMRGTFVVPL	10	HBV	adr	POL	1288	0.0020
1.0642	SLQLVFGIDV	10	MAGE	1		150	0.0020
1.0582	KLLHKRTLGL	10	HBV	adr	'X'	1509	0.0019
1.0713	GLGMEHLREV	10	c-ERB2			344	0.0017
1.0742	GMSYLEDVRL	10	c-ERB2			832	0.0017
1.0549	NLLSSNLSWL	10	HBV	adr	POL	991	0.0016
1.0465	QLTVWGIKQL	10	HIV		ENV	2760	0.0015
1.0524	VLEYLVSPGV	10	HBV	adr	CORE	505	0.0015
1.0483	VLNPSVAATL	10	HCV		LORF	1253	0.0015
1.0548	SLTNLLSSNL	10	HBV	adr	POL	988	0.0014
1.0512	ALLDPRVRGL	10	HBV	adr	ENV	119	0.0011
1.0676	TLEDSSGNLL	10	p53			256	0.0011
1.0719	TLQGLGISWL	10	c-ERB2			444	0.0011
1.0627	DLRAFQQFL	10	HPV	18	E7	82	0.0010
1.0725	VLQGLPREYV	10	c-ERB2			546	0.0009

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0918	DLPPWFPPMV	10	EBNA1			605	0.0009
1.0499	DLSDGSWSTV	10	HCV		LORF	2399	0.0008
1.0559	CLAFSYMDDV	10	HBV	adr	POL	1118	0.0008
1.0632	PLVLGTLEEV	10	MAGE	1		37	0.0008
1.0520	NLATWVGSNL	10	HBV	adr	CORE	457	0.0008
1.0400	NLLTQIGCTL	10	HIV		POL	684	0.0007
1.0488	GLTHIDAHFL	10	HCV		LORF	1564	0.0007
1.0733	VLGSGAFGTV	10	c-ERB2			725	0.0007
1.0434	QLIKKEKVYL	10	HIV		POL	1219	0.0006
1.0451	KLLWKGEHAV	10	HIV		POL	1495	0.0006
1.0470	SMVGNWAKVL	10	HCV		ENV1	364	0.0006
1.0570	KLIGTDNSVV	10	HBV	adr	POL	1317	0.0006
1.0924	ILLVVVLGVV	10	c-ERB2			661	0.0006
1.0397	LIDTGADDTV	10	HIV		POL	619	0.0005
1.0446	HLKTAVQMAV	10	HIV		POL	1426	0.0005
1.0604	DLLMGTLGIV	10	HPV	16	E7	81	0.0005
1.0443	LLKLAGRWPV	10	HIV		POL	1356	0.0004
1.0461	DLMVTVYYGV	10	HIV		ENV	2181	0.0004
1.0619	TLEKLTNTGL	10	HPV	18	E6	89	0.0004
1.0787	SLTNLLSSDL	10	HBV	adw	POL	1017	0.0004
1.0521	NLEDPASREL	10	HBV	adr	CORE	465	0.0003
1.0583	GLSAMSTTDL	10	HBV	adr	'X'	1517	0.0003
1.0652	VLVASRGRAV	10	PSA			36	0.0003
1.0716	DLSVFQNLQV	10	c-ERB2			421	0.0003

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0723	QLFRNPHQAL	10	c-ERB2			484	0.0003
1.0727	PLTSIISAVV	10	c-ERB2			650	0.0003
1.0479	YLKSSGGPL	10	HCV		LORF	1160	0.0002
1.0497	QLPCEPEPDV	10	HCV		LORF	2159	0.0002
1.0523	CLTFGRETVL	10	HBV	adr	CORE	497	0.0002
1.0603	TLEDLLMGTL	10	HPV	16	E7	78	0.0002
1.0631	SLHCKPEEAL	10	MAGE	1		7	0.0002
1.0680	EMFREINEAL	10	p53			339	0.0002
1.0689	VLKDAIKDLV	10	EBNA1			574	0.0002
1.0757	DLVDAEEYLV	10	c-ERB2			1016	0.0002
1.0796	RMRGTFVSPL	10	HBV	adw	POL	1317	0.0002
1.0669	QLAKTCPVQL	10	p53			136	0.0001
1.0717	NLQVIRGRIL	10	c-ERB2			427	0.0001
1.0721	WLGLRSLREL	10	c-ERB2			452	0.0001
1.0522	NMGLKIRQLL	10	HBV	adr	CORE	482	0
1.0527	PLSYQHFRKL	10	HBV	adr	POL	576	0
1.0529	ELPRLADEGL	10	HBV	adr	POL	598	0
1.0531	GLNRRVAEDL	10	HBV	adr	POL	606	0
1.0536	PLTVNEKRRL	10	HBV	adr	POL	672	0
1.0539	IMPARFYPNL	10	HBV	adr	POL	684	0
1.0550	PLHPAAMPHL	10	HBV	adr	POL	1012	0
1.0552	DLHDSCSRNL	10	HBV	adr	POL	1051	0
1.0555	LLYKTFGRKL	10	HBV	adr	POL	1066	0
1.0557	PMGVGLSPFL	10	HBV	adr	POL	1090	0

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0560	VLGAKSVQHL	10	HBV	adr	POL	1128	0
1.0569	PLPIHTAELL	10	HBV	adr	POL	1296	0
1.0579	PLPSLAFAV	10	HBV	adr	'X'	1454	0
1.0585	DLEAYFKDCL	10	HBV	adr	'X'	1525	0
1.0587	ELGEEIRLKV	10	HBV	adr	'X'	1540	0
1.0589	VLGGCRHKLV	10	HBV	adr	'X'	1551	0
1.0597	TLEQQYNKPL	10	HPV	16	E6	94	0
1.0608	DLCTELNTSL	10	HPV	18	E6	16	0
1.0616	RLQRRRETQV	10	HPV	18	E6	49	0
1.0621	HLEPQNEIPV	10	HPV	18	E7	14	0
1.0639	LLKYRAREPV	10	MAGE	1/3		114	0
1.0643	CLGLSYDGLL	10	MAGE	1/3		174	0
1.0657	DMSLLKNRFL	10	PSA			98	0
1.0658	LLRLSEPAEL	10	PSA			119	0
1.0663	PLSQETFSDL	10	p53			13	0
1.0664	PLPSQAMDDL	10	p53			34	0
1.0690	ELAALCRWGL	10	c-ERB2			2	0
1.0692	RLPASPETHL	10	c-ERB2			34	0
1.0699	RLRIVRGTOQL	10	c-ERB2			98	0
1.0701	GLRELQLRSL	10	c-ERB2			136	0
1.0730	QMRILKETEL	10	c-ERB2			711	0
1.0732	ILKETELRKV	10	c-ERB2			714	0
1.0754	PLDSTFYRSL	10	c-ERB2			999	0
1.0755	LLEDDDMGDL	10	c-ERB2			1008	0

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0758	DLGMGAAGKL	10	c-ERB2			1089	9
1.0761	PLPSETDGYV	10	c-ERB2			1119	0
1.0763	TLSPGKNGVV	10	c-ERB2			1172	0
1.0765	TLQDPRVRAL	10	HBV	adw	ENV	119	0
1.0768	NMENIASGLL	10	HBV	adw	ENV	163	0
1.0775	ELPHLADEGL	10	HBV	adw	POL	627	0
1.0776	GLNRPVAEDL	10	HBV	adw	POL	635	0
1.0777	PLTVNENRRL	10	HBV	adw	POL	701	0
1.0790	LLYKTYGRKL	10	HBV	adw	POL	1095	0
1.0801	GLSAMSPTDL	10	HBV	adw	"X"	1546	0
1.0802	DLEAYFKDCV	10	HBV	adw	"X"	1554	0
1.0803	TLQDPRVRGL	10	HBV	ayw	ENV	119	0
1.0804	NMENITSGFL	10	HBV	ayw	ENV	163	0
1.0891	DLVNLLPAIL	10	HCV		LORF	1878	0
1.0404	PLTEEKIKAL	10	HIV		POL	720	<0.0002
1.0409	QLGIPHPAGL	10	HIV		POL	786	<0.0002
1.0411	GLKKKKSVTV	10	HIV		POL	794	<0.0002
1.0450	PIWKGPAKLL	10	HIV		POL	1488	<0.0002
1.0476	DLAVAVEPVV	10	HCV		LORF	966	<0.0002
1.0478	SLTGRDKNQV	10	HCV		LORF	1046	<0.0002
1.0490	DLEVVTSTWV	10	HCV		LORF	1652	<0.0002
1.0494	GLGKVLIDIL	10	HCV		LORF	1843	<0.0002
1.0505	VLTTSCGNTL	10	HCV		LORF	2704	<0.0002
1.0506	ELITSCSSNV	10	HCV		LORF	2781	<0.0002

APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0510	CLRKLGVPL	10	HCV		LORF	2908	<0.0002
1.0511	PLGFFPDHQL	10	HBV	adr	ENV	10	<0.0002
1.0514	NMENTTSGFL	10	HBV	adr	ENV	163	<0.0002

Appendix III PLP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (EO2)
Hu PLP	10	8	C	L	V	G	A	P	F	A	
Hu PLP	13	8	G	A	P	F	A	S	L	V	
Hu PLP	23	8	G	L	C	F	F	G	V	A	
Hu PLP	39	8	A	L	T	G	T	E	K	L	
Hu PLP	40	8	L	T	G	T	E	K	L	I	
Hu PLP	60	8	Y	L	I	N	V	I	H	A	
Ms PLP	64	8	V	I	H	A	F	Q	C	V	
Hu PLP	64	8	V	I	H	A	F	Q	Y	V	
Hu PLP	74	8	G	T	A	S	F	F	F	L	
Hu PLP	80	8	F	L	Y	G	A	L	L	L	
Hu PLP	93	8	T	T	G	A	V	R	Q	I	
Hu PLP	106	8	T	T	I	C	G	K	G	L	
Hu PLP	131	8	Q	A	H	S	L	E	R	V	
Hu PLP	152	8	F	V	G	I	T	Y	A	L	
Hu PLP	154	8	G	I	T	Y	A	L	T	V	
Hu PLP	155	8	I	T	Y	A	L	T	V	V	
Hu PLP	157	8	Y	A	L	T	V	V	W	L	
Hu PLP	158	8	A	L	T	V	V	W	L	L	
Hu PLP	159	8	L	T	V	V	W	L	L	V	
Hu PLP	164	8	L	L	V	F	A	C	S	A	
Hu PLP	165	8	L	V	F	A	C	S	A	V	
Hu PLP	167	8	F	A	C	S	A	V	P	V	
Hu PLP	199	8	S	L	C	A	D	A	R	M	
Hu PLP	203	8	D	A	R	M	Y	G	V	L	
Hu PLP	212	8	W	I	A	F	P	G	K	V	
Hu PLP	218	8	K	V	C	G	S	N	L	L	
Hu PLP	224	8	L	L	S	I	C	K	T	A	
Hu PLP	234	8	Q	M	T	F	H	L	F	I	
Hu PLP	238	8	H	L	F	I	A	A	F	V	
Hu PLP	244	8	F	V	G	A	A	A	T	L	
Hu PLP	247	8	A	A	A	T	L	V	S	L	

Appendix III PLP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (EO2)
Hu PLP	248	8	A	A	T	L	V	S	L	L	
Hu PLP	253	8	S	L	L	T	F	M	I	A	
Hu PLP	254	8	L	L	T	F	M	I	A	A	
Hu PLP	260	8	A	A	T	Y	N	F	A	V	
Hu PLP	261	8	A	T	Y	N	F	A	V	L	

Appendix III MBP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (E02)
Hu MBP	14	8	Y	L	A	T	A	S	T	M	
Hu MBP	34	8	D	T	G	I	L	D	S	I	
Hu MBP	65	8	R	T	A	H	Y	G	S	L	
Ms MBP	70	8	H	A	R	S	R	P	G	L	
Hu MBP	79	8	R	T	Q	D	E	N	P	V	
Hu MBP	86	8	V	V	H	F	F	K	N	I	
Ms MBP	87	8	R	T	T	H	Y	G	S	L	
Hu MBP	143	8	G	V	D	A	Q	G	T	L	
Hu MBP	149	8	T	L	S	K	I	F	K	L	

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (EO2)
Hu PLP	163	9	W	L	L	V	F	A	C	S	A	-18.67
Hu PLP	205	9	R	M	Y	G	V	L	P	W	I	-18.79
Hu PLP	145	9	W	L	G	H	P	D	K	F	V	-19.05
Hu PLP	253	9	S	L	L	T	F	M	I	A	A	-19.07
Hu PLP	251	9	L	V	S	L	L	T	F	M	I	-20.03
Hu PLP	258	9	M	I	A	A	T	Y	N	F	A	-20.32
Hu PLP	80	9	F	L	Y	G	A	L	L	L	A	-20.53
Ms PLP	205	9	R	M	Y	G	V	L	P	W	N	-20.69
Hu PLP	64	9	V	I	H	A	F	Q	Y	V	I	-20.71
Hu PLP	23	9	G	L	C	F	F	G	V	A	L	-21.23
Ms PLP	23	9	G	L	C	F	F	G	V	A	L	-21.23
Ms PLP	179	9	N	T	W	T	T	C	Q	S	I	-21.24
Hu PLP	233	9	F	Q	M	T	F	H	L	F	I	-21.25
Hu PLP	234	9	Q	M	T	F	H	L	F	I	A	-21.29
Hu PLP	259	9	I	A	A	T	Y	N	F	A	V	-21.32
Hu PLP	157	9	Y	A	L	T	V	V	W	L	L	-21.51
Hu PLP	76	9	A	S	F	F	F	L	Y	G	A	-21.52
Hu PLP	158	9	A	L	T	V	V	W	L	L	V	-21.56
Hu PLP	252	9	V	S	L	L	T	F	M	I	A	-21.58
Hu PLP	237	9	F	H	L	F	I	A	A	F	V	-21.61
Ms PLP	208	9	G	V	L	P	W	N	A	F	P	-21.61
Hu PLP	164	9	L	L	V	F	A	C	S	A	V	-21.81
Hu PLP	78	9	F	F	F	L	Y	G	A	L	L	-22.05
Hu PLP	250	9	T	L	V	S	L	L	T	F	M	-22.10
Hu PLP	208	9	G	V	L	P	W	I	A	F	P	-22.10
Hu PLP	39	9	A	L	T	G	T	E	K	L	I	-22.13
Hu PLP	240	9	F	I	A	A	F	V	G	A	A	-22.19
Hu PLP	235	9	M	T	F	H	L	F	I	A	A	-22.22
Hu PLP	244	9	F	V	G	A	A	A	T	L	V	-22.22
Ms PLP	64	9	V	I	H	A	F	Q	C	V	I	-22.33

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (E02)
Hu PLP	12	9	V	G	A	P	F	A	S	L	V	-22.36
Hu PLP	45	9	K	L	I	E	T	Y	F	S	K	-22.42
Hu PLP	30	9	A	L	F	C	G	C	G	H	E	-22.46
Hu PLP	9	9	R	C	L	V	G	A	P	F	A	-22.52
Hu PLP	189	9	F	P	S	K	T	S	A	S	I	-22.54
Hu PLP	71	9	V	I	Y	G	T	A	S	F	F	-22.60
Hu PLP	73	9	Y	G	T	A	S	F	F	F	L	-22.63
Hu PLP	11	9	L	V	G	A	P	F	A	S	L	-22.64
Hu PLP	86	9	L	L	A	E	G	F	Y	T	T	-22.65
Ms PLP	63	9	N	V	I	H	A	F	Q	C	V	-22.65
Hu PLP	212	9	W	I	A	F	P	G	K	V	C	-22.67
Hu PLP	223	9	N	L	L	S	I	C	K	T	A	-22.68
Hu PLP	199	9	S	L	C	A	D	A	R	M	Y	-22.71
Hu PLP	179	9	N	T	W	T	T	C	D	S	I	-22.73
Hu PLP	201	9	C	A	D	A	R	M	Y	G	V	-22.74
Hu PLP	112	9	G	L	S	A	T	V	T	G	G	-22.78
Hu PLP	161	9	V	V	W	L	L	V	F	A	C	-22.78
Hu PLP	175	9	Y	I	Y	F	N	T	W	T	T	-22.81

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (E02)
Hu PLP	56	9	Q	D	Y	E	Y	L	I	N	V	-22.84
Hu PLP	241	9	I	A	A	F	V	G	A	A	A	-22.87
Hu PLP	154	9	G	I	T	Y	A	L	T	V	V	-22.89
Hu PLP	257	9	F	M	I	A	A	T	Y	N	F	-22.89
Hu PLP	196	9	S	I	G	S	L	C	A	D	A	-22.90
Hu PLP	18	9	S	L	V	A	T	G	L	C	F	-22.91
Hu PLP	261	9	A	T	Y	N	F	A	V	L	K	-23.00
Hu PLP	171	9	A	V	P	V	Y	I	Y	F	N	-23.05
Hu PLP	70	9	Y	V	I	Y	G	T	A	S	F	-23.11
Hu PLP	22	9	T	G	L	C	F	F	G	V	A	-23.12
Hu PLP	134	9	S	L	E	R	V	C	H	C	L	-23.16
Hu PLP	16	9	F	A	S	L	V	A	T	G	L	-23.20
Hu PLP	74	9	G	T	A	S	F	F	F	L	Y	-23.20
Hu PLP	79	9	F	F	L	Y	G	A	L	L	L	-23.24
Hu PLP	246	9	G	A	A	A	T	L	V	S	L	-23.26
Hu PLP	181	9	W	T	T	C	D	S	I	A	F	-23.27
Hu PLP	28	9	G	V	A	L	F	C	G	C	G	-23.31
Hu PLP	247	9	A	A	A	T	L	V	S	L	L	-23.31
Hu PLP	219	9	V	C	G	S	N	L	L	S	I	-23.33
Hu PLP	160	9	T	V	V	W	L	L	V	F	A	-23.40
Hu PLP	54	9	N	Y	Q	D	Y	E	Y	L	I	-23.43
Hu PLP	107	9	T	I	C	G	K	G	L	S	A	-23.45
Hu PLP	166	9	V	F	A	C	S	A	V	P	V	-23.53
Hu PLP	2	9	G	L	L	E	C	C	A	R	C	-23.57
Hu PLP	167	9	F	A	C	S	A	V	P	V	Y	-23.60
Hu PLP	260	9	A	A	T	Y	N	F	A	V	L	-23.61
Hu PLP	152	9	F	V	G	I	T	Y	A	L	T	-23.63
Hu PLP	187	9	I	A	F	P	S	K	T	S	A	-23.64
Hu PLP	63	9	N	V	I	H	A	F	Q	Y	V	-23.65
Hu PLP	60	9	Y	L	I	N	V	I	H	A	F	-23.66
Hu PLP	85	9	L	L	L	A	E	G	F	Y	T	-23.66

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (EO2)
Ms PLP	210	9	L	P	W	N	A	F	P	G	K	-23.66
Hu PLP	198	9	G	S	L	C	A	D	A	R	M	-23.67
Hu PLP	20	9	V	A	T	G	L	C	F	F	G	-23.71
Hu PLP	263	9	Y	N	F	A	V	L	K	L	M	-23.71
Ms PLP	209	9	V	L	P	W	N	A	F	P	G	-23.71
Hu PLP	84	9	A	L	L	L	A	E	G	F	Y	-23.73
Hu PLP	206	9	M	Y	G	V	L	P	W	I	A	-23.77
Hu PLP	153	9	V	G	I	T	Y	A	L	T	V	-23.80
Hu PLP	269	9	K	L	M	G	R	G	T	K	F	-23.92
Hu PLP	138	9	V	C	H	C	L	G	K	W	L	-23.99
Hu PLP	3	9	L	L	E	C	C	A	R	C	L	-24.02
Hu PLP	92	9	Y	T	T	G	A	V	R	Q	I	-24.40
Hu PLP	21	9	A	T	G	L	C	F	F	G	V	-24.47
Hu PLP	192	9	K	T	S	A	S	I	G	S	L	-24.74
Hu PLP	38	9	E	A	L	T	G	T	E	K	L	-25.72
Hu PLP	105	9	K	T	T	I	C	G	K	G	L	-26.97

Appendix III MBP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (E02)
Hu MBP	110	9	S	L	S	R	F	S	W	G	A	-21.42
Hu MBP	14	9	Y	L	A	T	A	S	T	M	D	-22.01
Ms MBP	59	9	W	L	K	Q	S	R	S	P	L	-22.60
Hu MBP	86	9	V	V	H	F	F	K	N	I	V	-22.80
Ms MBP	52	9	R	G	S	G	K	V	P	W	L	-22.87
Hu MBP	16	9	A	T	A	S	T	M	D	H	A	-23.11
Hu MBP	37	9	I	L	D	S	I	G	R	F	F	-23.11
Hu MBP	108	9	G	L	S	L	S	R	F	S	W	-23.34
Hu MBP	93	9	I	V	T	P	R	T	P	P	P	-23.41
Ms MBP	63	9	S	R	S	P	L	P	S	H	A	-23.47
Hu MBP	79	9	R	T	Q	D	E	N	P	V	V	-23.49
Hu MBP	129	9	G	R	A	S	D	Y	K	S	A	-23.53
Hu MBP	21	9	M	D	H	A	R	H	G	F	L	-23.60
Hu MBP	160	9	D	S	R	S	G	S	P	M	A	-23.63
Ms MBP	75	9	P	G	L	C	H	M	Y	K	D	-23.64
Hu MBP	112	9	S	R	F	S	W	G	A	E	G	-23.77
Hu MBP	162	9	R	S	G	S	P	M	A	R	R	-23.77
Hu MBP	159	9	R	D	S	R	S	G	S	P	M	-23.81
Hu MBP	85	9	P	V	V	H	F	F	K	N	I	-23.82
Hu MBP	136	9	S	A	H	K	G	F	K	G	V	-23.90
Hu MBP	149	9	T	L	S	K	I	F	K	L	G	-23.90
Ms MBP	162	9	K	G	F	K	G	A	Y	D	A	-23.92
Hu MBP	64	9	A	R	T	A	H	Y	G	S	L	-23.99
Ms MBP	166	9	G	A	Y	D	A	Q	G	T	L	-24.66
Hu MBP	148	9	G	T	L	S	K	I	F	K	L	-24.78
Hu MBP	145	9	D	A	Q	G	T	L	S	K	I	-25.25

Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (EO2)
Ms PLP	178	10	F	N	T	W	T	T	C	Q	S	I	-24.68
Hu PLP	178	10	F	N	T	W	T	T	C	D	S	I	-25.14
Hu PLP	204	10	A	R	M	Y	G	V	L	P	W	I	-25.48
Hu PLP	163	10	W	L	L	V	F	A	C	S	A	V	-25.66
Hu PLP	218	10	K	V	C	G	S	N	L	L	S	I	-25.89
Hu PLP	250	10	T	L	V	S	L	L	T	F	M	I	-26.00
Hu PLP	19	10	L	V	A	T	G	L	C	F	F	G	-26.25
Hu PLP	78	10	F	F	F	L	Y	G	A	L	L	L	-26.68
Hu PLP	157	10	Y	A	L	T	V	V	W	L	L	V	-26.72
Hu PLP	84	10	A	L	L	L	A	E	G	F	Y	T	-26.77
Hu PLP	233	10	F	Q	M	T	F	H	L	F	I	A	-26.78
Hu PLP	80	10	F	L	Y	G	A	L	L	L	A	E	-26.79
Hu PLP	167	10	F	A	C	S	A	V	P	V	Y	I	-27.28
Hu PLP	165	10	L	V	F	A	C	S	A	V	P	V	-27.32
Hu PLP	4	10	L	E	C	C	A	R	C	L	V	G	-27.36
Hu PLP	253	10	S	L	L	T	F	M	I	A	A	T	-27.42
Hu PLP	135	10	L	E	R	V	C	H	C	L	G	K	-27.48
Hu PLP	176	10	I	Y	F	N	T	W	T	T	C	D	-27.62
Hu PLP	24	10	L	C	F	F	G	V	A	L	F	C	-27.74
Hu PLP	146	10	L	G	H	P	D	K	F	V	G	I	-27.88
Hu PLP	237	10	F	H	L	F	I	A	A	F	V	G	-27.95
Hu PLP	56	10	Q	D	Y	E	Y	L	I	N	V	I	-27.99
Ms PLP	204	10	A	R	M	Y	G	V	L	P	W	N	-28.01
Hu PLP	158	10	A	L	T	V	V	W	L	L	V	F	-28.04
Hu PLP	137	10	R	V	C	H	C	L	G	K	W	L	-28.15
Hu PLP	72	10	I	Y	G	T	A	S	F	F	F	L	-28.16
Hu PLP	63	10	N	V	I	H	A	F	Q	Y	V	I	-28.17
Hu PLP	208	10	G	V	L	P	W	I	A	F	P	G	-28.17
Hu PLP	27	10	F	G	V	A	L	F	C	G	C	G	-28.29
Hu PLP	85	10	L	L	L	A	E	G	F	Y	T	T	-28.32
Ms PLP	62	10	I	N	V	I	H	A	F	Q	C	V	-28.33

Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (E02)
Hu PLP	222	10	S	N	L	L	S	I	C	K	T	A	-28.40
Hu PLP	76	10	A	S	F	F	F	L	Y	G	A	L	-28.43
Ms PLP	208	10	G	V	L	P	W	N	A	F	P	G	-28.45
Hu PLP	207	10	Y	G	V	L	P	W	I	A	F	P	-28.46
Hu PLP	79	10	F	F	L	Y	G	A	L	L	L	A	-28.49
Hu PLP	236	10	T	F	H	L	F	I	A	A	F	V	-28.50
Hu PLP	240	10	F	I	A	A	F	V	G	A	A	A	-28.51
Hu PLP	181	10	W	T	T	C	D	S	I	A	F	P	-28.56
Hu PLP	224	10	L	L	S	I	C	K	T	A	E	F	-28.56
Hu PLP	10	10	C	L	V	G	A	P	F	A	S	L	-28.62
Hu PLP	152	10	F	V	G	I	T	Y	A	L	T	V	-28.64
Hu PLP	62	10	I	N	V	I	H	A	F	Q	Y	V	-28.64
Hu PLP	214	10	A	F	P	G	K	V	C	G	S	N	-28.65
Hu PLP	188	10	A	F	P	S	K	T	S	A	S	I	-28.65
Hu PLP	99	10	Q	I	F	G	D	Y	K	T	T	I	-28.69
Hu PLP	18	10	S	L	V	A	T	G	L	C	F	F	-28.73
Hu PLP	3	10	L	L	E	C	C	A	R	C	L	V	-28.75

Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (E02)
Hu PLP	17	10	A	S	L	V	A	T	G	L	C	F	-28.76
Hu PLP	144	10	K	W	L	G	H	P	D	K	F	V	-28.78
Ms PLP	181	10	W	T	T	C	Q	S	I	A	F	P	-28.78
Hu PLP	159	10	L	T	V	V	W	L	L	V	F	A	-28.79
Hu PLP	174	10	V	Y	I	Y	F	N	T	W	T	T	-28.80
Hu PLP	248	10	A	A	T	L	V	S	L	L	T	F	-28.84
Hu PLP	23	10	G	L	C	F	F	G	V	A	L	F	-28.87
Hu PLP	209	10	V	L	P	W	I	A	F	P	G	K	-28.87
Hu PLP	29	10	V	A	L	F	C	G	C	G	H	E	-28.90
Hu PLP	261	10	A	T	Y	N	F	A	V	L	K	L	-28.92
Ms PLP	63	10	N	V	I	H	A	F	Q	C	V	I	-28.93
Hu PLP	74	10	G	T	A	S	F	F	F	L	Y	G	-28.93
Hu PLP	259	10	I	A	A	T	Y	N	F	A	V	L	-29.06
Hu PLP	242	10	A	A	F	V	G	A	A	A	T	L	-29.24
Hu PLP	2	10	G	L	L	E	C	C	A	R	C	L	-29.30
Hu PLP	257	10	F	M	I	A	A	T	Y	N	F	A	-29.37
Hu PLP	20	10	V	A	T	G	L	C	F	F	G	V	-29.41
Ms PLP	205	10	R	M	Y	G	V	L	P	W	N	A	-29.43
Hu PLP	155	10	I	T	Y	A	L	T	V	V	W	L	-29.60
Hu PLP	30	10	A	L	F	C	G	C	G	H	E	A	-29.70
Hu PLP	205	10	R	M	Y	G	V	L	P	W	I	A	-29.74
Hu PLP	258	10	M	I	A	A	T	Y	N	F	A	V	-30.06
Hu PLP	234	10	Q	M	T	F	H	L	F	I	A	A	-30.29
Hu PLP	238	10	H	L	F	I	A	A	F	V	G	A	-30.64
Hu PLP	246	10	G	A	A	A	T	L	V	S	L	L	-30.64
Hu PLP	38	10	E	A	L	T	G	T	E	K	L	I	-30.92
Hu PLP	230	10	T	A	E	F	Q	M	T	F	H	L	-31.03
Hu PLP	11	10	L	V	G	A	P	F	A	S	L	V	-31.25
Hu PLP	201	10	C	A	D	A	R	M	Y	G	V	L	-31.73

Appendix III PLP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu PLP	2	11	G	L	L	E	C	C	A	R	C	L	V	
Hu PLP	10	11	C	L	V	G	A	P	F	A	S	L	V	
Hu PLP	19	11	L	V	A	T	G	L	C	F	F	G	V	
Hu PLP	21	11	A	T	G	L	C	F	F	G	V	A	L	
Hu PLP	30	11	A	L	F	C	G	C	G	H	E	A	L	
Hu PLP	61	11	L	I	N	V	I	H	A	F	Q	Y	V	
Ms PLP	61	11	L	I	N	V	I	H	A	F	Q	C	V	
Hu PLP	71	11	V	I	Y	G	T	A	S	F	F	F	L	
Hu PLP	75	11	T	A	S	F	F	F	L	Y	G	A	L	
Hu PLP	86	11	L	L	A	E	G	F	Y	T	T	G	A	
Hu PLP	87	11	L	A	E	G	F	Y	T	T	G	A	V	
Hu PLP	107	11	T	I	C	G	K	G	L	S	A	T	V	
Hu PLP	145	11	W	L	G	H	P	D	K	F	V	G	I	
Hu PLP	152	11	F	V	G	I	T	Y	A	L	T	V	V	
Hu PLP	154	11	G	I	T	Y	A	L	T	V	V	W	L	
Hu PLP	155	11	I	T	Y	A	L	T	V	V	W	L	L	
Hu PLP	158	11	A	L	T	V	V	W	L	L	V	F	A	
Hu PLP	164	11	L	L	V	F	A	C	S	A	V	P	V	
Hu PLP	187	11	I	A	F	P	S	K	T	S	A	S	I	
Hu PLP	199	11	S	L	C	A	D	A	R	M	Y	G	V	
Hu PLP	203	11	D	A	R	M	Y	G	V	L	P	W	I	
Hu PLP	209	11	V	L	P	W	I	A	F	P	G	K	V	
Ms PLP	209	11	V	L	P	W	N	A	F	P	G	K	V	
Hu PLP	229	11	K	T	A	E	F	Q	M	T	F	H	L	
Hu PLP	235	11	M	T	F	H	L	F	I	A	A	F	V	
Hu PLP	238	11	H	L	F	I	A	A	F	V	G	A	A	
Hu PLP	241	11	I	A	A	F	V	G	A	A	A	T	L	
Hu PLP	242	11	A	A	F	V	G	A	A	A	T	L	V	
Hu PLP	244	11	F	V	G	A	A	A	T	L	V	S	L	
Hu PLP	249	11	A	T	L	V	S	L	L	T	F	M	I	

Appendix III PLP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu PLP	250	11	T	L	V	S	L	L	T	F	M	I	A	
Hu PLP	257	11	F	M	I	A	A	T	Y	N	F	A	V	
Hu PLP	258	11	M	I	A	A	T	Y	N	F	A	V	L	
Hu PLP	260	11	A	A	T	Y	N	F	A	V	L	K	L	

Appendix III MBP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (E02)
Hu MBP	37	10	I	L	D	S	I	G	R	F	F	G	-27.66
Hu MBP	28	10	F	L	P	R	H	R	D	T	G	I	-27.85
Ms MBP	167	10	A	Y	D	A	Q	G	T	L	S	K	-28.54
Hu MBP	89	10	F	F	K	N	I	V	T	P	R	T	-28.68
Hu MBP	14	10	Y	L	A	T	A	S	T	M	D	H	-28.75
Hu MBP	84	10	N	P	V	V	H	F	F	K	N	I	-28.80
Hu MBP	32	10	H	R	D	T	G	I	L	D	S	I	-28.83
Hu MBP	110	10	S	L	S	R	F	S	W	G	A	E	-28.98
Hu MBP	85	10	P	V	V	H	F	F	K	N	I	V	-30.82
Ms MBP	85	10	H	T	R	T	T	H	Y	G	S	L	-31.29
Hu MBP	20	10	T	M	D	H	A	R	H	G	F	L	-31.40
Hu MBP	63	10	P	A	R	T	A	H	Y	G	S	L	-31.76
Ms MBP	48	10	G	A	P	K	R	G	S	G	K	V	-32.21

Appendix III MBP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu MBP	14	11	Y	L	A	T	A	S	T	M	D	H	A	
Hu MBP	19	11	S	T	M	D	H	A	R	H	G	F	L	
Hu MBP	28	11	F	L	P	R	H	R	D	T	G	I	L	
Hu MBP	108	11	G	L	S	L	S	R	F	S	W	G	A	
Hu MBP	143	11	G	V	D	A	Q	G	T	L	S	K	I	

WHAT IS CLAIMED IS:

1. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide
5 has 9 residues and the following residues:

a first conserved residue at the second position from the N-terminus selected from the group consisting of I, V, A and T;

10 a second conserved residue at the C-terminal position selected from the group consisting of V, L, I, A and M.

2. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide
15 has 9 residues:

a first conserved residue at the second position from the N-terminus selected from the group consisting of L, M, I, V, A and T;

20 a second conserved residue at the C-terminal position selected from the group consisting of A and M;

3. The composition of claim 1, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, and P.
25

4. The composition of claim 2, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, and P.

30 5. The composition of claim 1, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H.

35 6. The composition of claim 2, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H

7. The composition of claim 1, wherein the amino acid at position 6 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

5 8. The composition of claim 2, wherein the amino acid at position 6 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

10 9. The composition of claim 1, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K, H, D and E.

15 10. The composition of claim 2, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K, H, D and E.

11. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide has about 10 residues:

20 a first conserved residue at the second position from the N-terminus selected from the group consisting of L, M, I, V, A, and T; and

25 a second conserved residue at the C-terminal position selected from the group consisting of V, I, L, A and M;

wherein the first and second conserved residues are separated by 7 residues.

30 12. The composition of claim 11, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, E and P.

35 13. The composition of claim 11, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D and E.

14. The composition of claim 11, wherein the amino acid at position 4 from the N-terminus is not an amino acid selected from the group consisting of A, K, R and H.

5 15. The composition of claim 11, wherein the amino acid at position 5 from the N-terminus is not P.

10 16. The composition of claim 11, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

15 17. The composition of claim 11, wherein the amino acid at position 8 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H.

18. The composition of claim 11, wherein the amino acid at position 9 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

20 19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a peptide capable of binding an HLA-A2.1 molecule and inducing an immune response in a mammal.

25 20. The pharmaceutical composition of claim 19, wherein the peptide has a formula as follows: TLGIVCPI.

30 21. The pharmaceutical composition of claim 19, further comprising a peptide having a formula as follows: YMLDLQPETT.

22. The pharmaceutical composition of claim 19, further comprising a T helper peptide.

35 23. The pharmaceutical composition of claim 22, wherein the T helper peptide has a formula as follows: aKXVAAWTLKAAa, wherein a is D-alanine and X is cyclohexylalanine.

1/3

HLA-A PURIFICATION AND
PEPTIDE ELUTION

CELLULAR SOURCE: HLA-ANTIGENS
($5-10 \times 10^9$ CELL EQUIVALENTS)

- A) EBV TRANSFORMED B CELL
LINES - HOMOZYGOUS
B) HLA-A TRANSFECTANTS -
e.g. .221-HLA-A1
C) P815 TRANSFECTANTS
(MOUSE MASTOCYTOMA)

↓
DETERGENT LYSIS
(10^8 CELLS/ml)

1% NP-40 OR 1% RENEX 30 PLUS
PROTEASE INHIBITORS - 1 HR, 4°C

↓
DETERGENT LYSATE

CENTRIFUGATION AT $15,000 \times g$,
30 MIN.

↓
AFFINITY CHROMATOGRAPHY

mAb-SEPHAROSE 5 mg/ml
5-10 ml COLUMN

↓
PURIFIED HLA-A ANTIGEN

ANTICIPATED YIELDS 450-900 μg

↓
ACID TREATMENT

10% ACETIC ACID, 5 MIN, 100°C

↓
PEPTIDES

YM3 FILTRATION, 3kD CUT-OFF

↓
SEQUENCE/MOTIF

D. HUNT - HPLC/EI-TMS
CYTEL - HPLC/ABI 477A

FIG. 1.

2/3

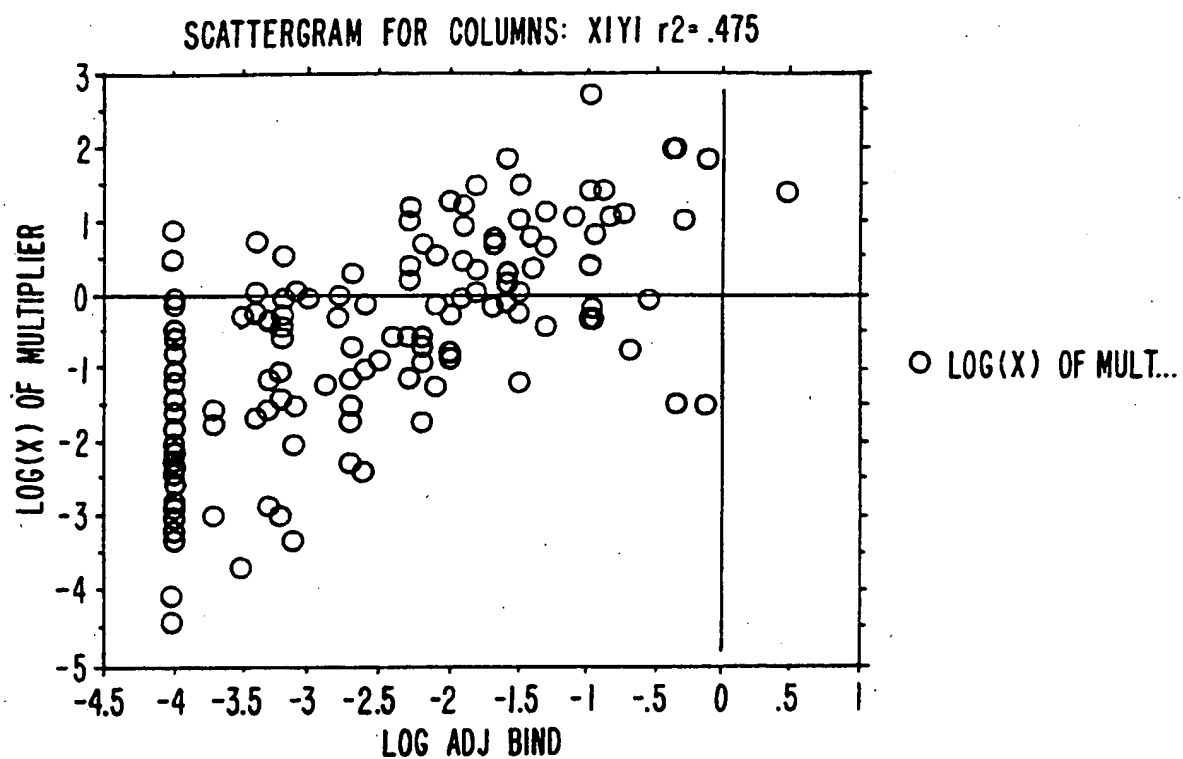


FIG. 2.

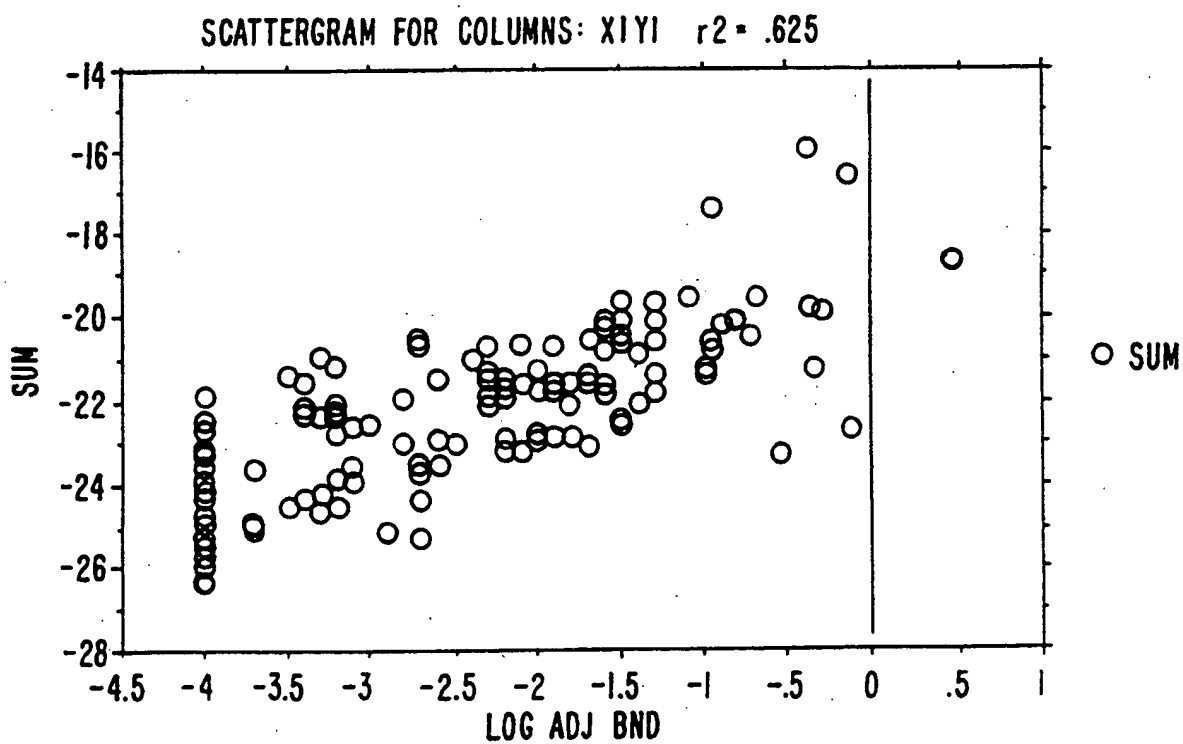


FIG. 3.

3/3

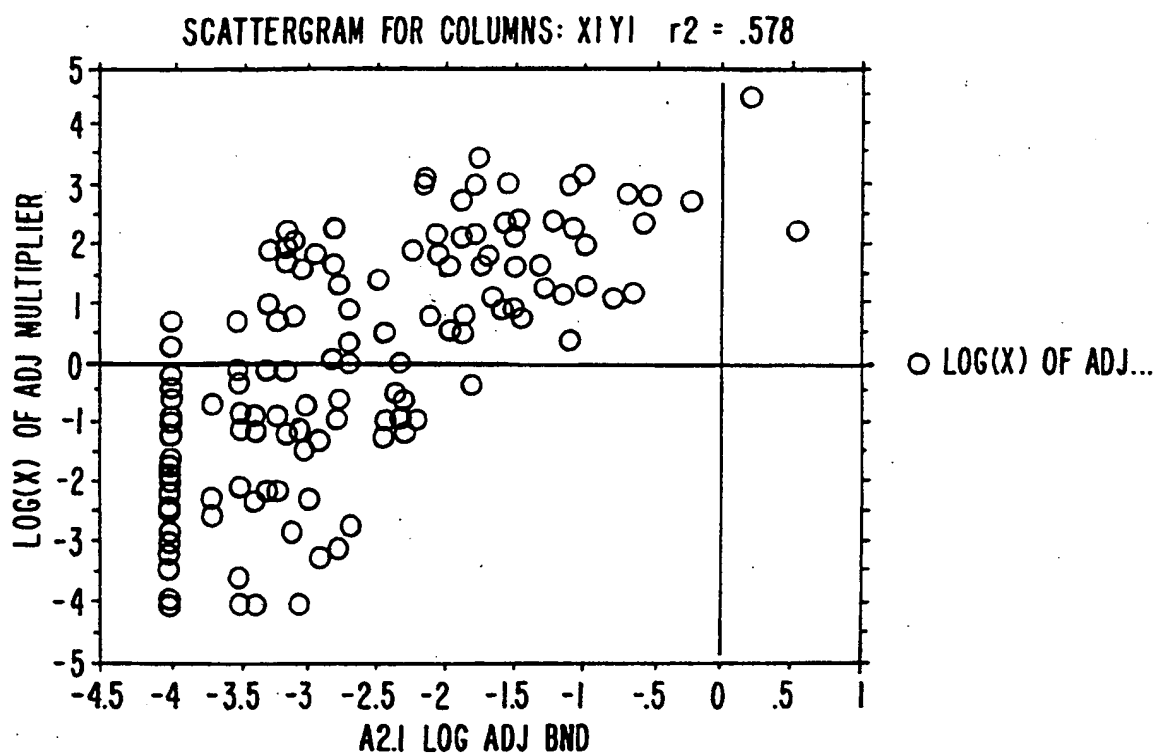


FIG. 4.

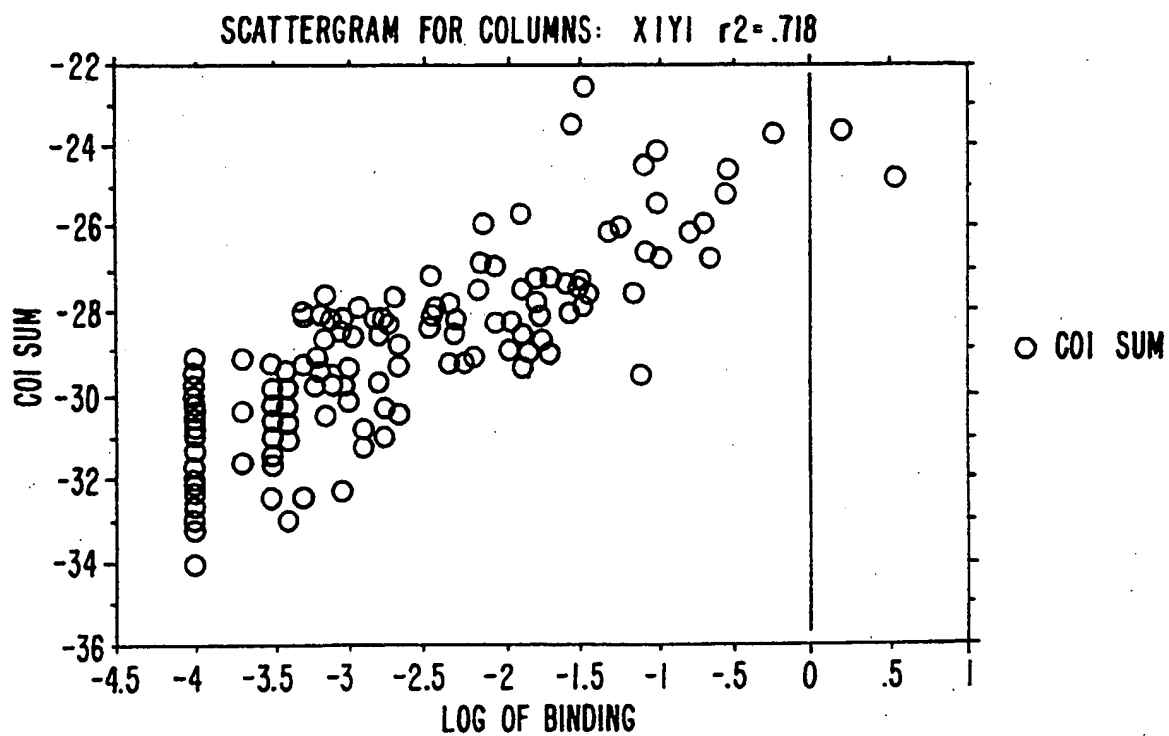


FIG. 5.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: HLA BINDING PEPTIDES AND THEIR USES (57) Abstract The present invention provides peptide compositions capable of specifically binding selected MHC alleles and inducing T cell activation in T cells restricted by the MHC allele. The peptides are useful to elicit an immune response against a desired antigen.		

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HLA BINDING PEPTIDES AND THEIR USES

The present application is a continuation in part of USSN 08/027,746 which is a continuation in part of USSN 07/926,666, which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10

The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as viral diseases and cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and inducing an immune response.

15

MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed. Class I MHC molecules are expressed on almost all nucleated cells and are recognized by cytotoxic T lymphocytes (CTLs), which then destroy the antigen-bearing cells. CTLs are particularly important in tumor rejection and in fighting viral infections. The CTL recognizes the antigen in the form of a peptide fragment bound to the MHC class I molecules rather than the intact foreign antigen itself. The antigen must normally be endogenously synthesized by the cell, and a portion of the protein antigen is degraded into small peptide fragments in the cytoplasm. Some of these small peptides translocate into a pre-Golgi compartment and interact with class I heavy chains to facilitate proper folding and association with the subunit B2 microglobulin. The peptide-MHC class I complex is then routed to the cell surface for expression and potential recognition by specific CTLs.

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Investigations of the crystal structure of the human MHC class I molecule, HLA-A2.1, indicate that a peptide binding groove is created by the folding of the $\alpha 1$ and $\alpha 2$ domains of the class I heavy chain (Bjorkman et al., Nature 329:506 (1987)). In these investigations, however, the identity of peptides bound to the groove was not determined.

Buus et al., Science 242:1065 (1988) first described a method for acid elution of bound peptides from MHC.

Subsequently, Rammensee and his coworkers (Falk et al., Nature 351:290 (1991)) have developed an approach to characterize naturally processed peptides bound to class I molecules.

Other investigators have successfully achieved direct amino acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from class I molecules of the B type (Jardetzky, et al., Nature 353:326 (1991)) and of the A2.1 type by mass spectrometry (Hunt, et al., Science 225:1261 (1992)). A review of the characterization of naturally processed peptides in MHC Class I has been presented by Rötzschke and Falk (Rötzschke and Falk, Immunol. Today 12:447 (1991)).

Sette et al., Proc. Natl. Acad. Sci. USA 86:3296 (1989) showed that MHC allele specific motifs could be used to predict MHC binding capacity. Schaeffer et al., Proc. Natl. Acad. Sci. USA 86:4649 (1989) showed that MHC binding was related to immunogenicity. Several authors (De Bruijn et al., Eur. J. Immunol., 21:2963-2970 (1991); Pamer et al., 1991 Nature 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Class I motifs specific for a number of human alleles of a given class I isotype have yet to be described. It is desirable that the combined frequencies of these different alleles should be high enough to cover a large fraction or perhaps the majority of the human outbred population.

Despite the developments in the art, the prior art has yet to provide a useful human peptide-based vaccine or therapeutic agent based on this work. The present invention provides these and other advantages.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising immunogenic peptides having binding motifs for MHC Class I molecules. The immunogenic peptides are typically between about 8 and about 11 residues and comprise conserved residues involved in binding proteins encoded by the appropriate MHC allele. A number of allele specific motifs have been identified.

For instance, the motif for HLA-A3.2 comprises from the N-terminus to C-terminus a first conserved residue of L, M, I, V, S, A, T and F at position 2 and a second conserved residue of K, R or Y at the C-terminal end. Other first conserved residues are C, G or D and alternatively E. Other second conserved residues are H or F. The first and second conserved residues are preferably separated by 6 to 7 residues.

The motif for HLA-A1 comprises from the N-terminus to the C-terminus a first conserved residue of T, S or M, a second conserved residue of D or E, and a third conserved residue of Y. Other second conserved residues are A, S or T. The first and second conserved residues are adjacent and are preferably separated from the third conserved residue by 6 to 7 residues. A second motif consists of a first conserved residue of E or D and a second conserved residue of Y where the first and second conserved residues are separated by 5 to 6 residues.

The motif for HLA-A11 comprises from the N-terminus to the C-terminus a first conserved residue of T or V at position 2 and a C-terminal conserved residue of K. The first and second conserved residues are preferably separated by 6 or 7 residues.

The motif for HLA-A24.1 comprises from the N-terminus to the C-terminus a first conserved residue of Y, F or W at position 2 and a C terminal conserved residue of F, I, W, M or L. The first and second conserved residues are preferably separated by 6 to 7 residues.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable

antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, malignant melanoma antigen (MAGE-1) Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. The peptides are thus useful in pharmaceutical compositions for both in vivo and ex vivo therapeutic and diagnostic applications.

Definitions

The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The oligopeptides of the invention are less than about 15 residues in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues.

An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will bind the MHC allele and be capable of inducing a CTL response. Thus, immunogenic peptides are capable of binding to an appropriate class I MHC molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

A "conserved residue" is an amino acid which occurs in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide motif. Typically a conserved residue is one at which the immunogenic peptide may provide a contact point with the MHC molecule. One to three, preferably two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself. Typically, an immunogenic peptide will comprise up to three conserved residues, more usually two conserved residues.

As used herein, "negative binding residues" are amino acids which if present at certain positions will result

in a peptide being a nonbinder or poor binder and in turn fail to induce a CTL response despite the presence of the appropriate conserved residues within the peptide.

5 The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 8 to about 11 amino acids, which is recognized by a particular MHC allele. The peptide motifs are typically different for each human MHC allele and differ in the pattern of the highly conserved residues.

10 The binding motif for an allele can be defined with increasing degrees of precision. In one case, all of the conserved residues are present in the correct positions in a peptide and there are no negative binding residues present.

15 The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their in situ environment, e.g., MHC I molecules on antigen presenting cells. Even where
20 a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

25 The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification scheme.

5 Fig. 2 is an SDS-PAGE analysis of affinity purified. HLA-A3.2 from the cell line EHM using an affinity column prepared with the mAb GAP A3 coupled to protein A-Sepharose.

Lane 1 - Molecular weight standards.

Lane 2 - A3.2 acid eluate

10 Lane 3 - A3.2 a second acid eluate

Lane 4 - Base elution #1

Lane 5 - Base elution #2

Lane 6 - Concentrated base elution 1

Lane 7 - Concentrated base elution 2

15 Lane 8 - BSA - 10 μ g

Lane 9 - BSA - 3 μ g

Lane 10 - BSA - 1 μ g

20 Fig. 3 shows reverse phase high performance liquid chromatography (RP-HPLC) separation of HLA-A3 acid eluted peptides.

Fig. 4 shows binding of a radioactively labeled peptide of the invention to MHC molecules as measured by the % bound radioactivity.

25 Fig. 5 shows inhibition of binding of a peptide of the invention to MHC molecules in the presence of three peptides [HBC 18-27 (924.07), a Prostate Specific Antigen peptide (939.01), and HIV nef 73-82 (940.03)].

Fig. 6 shows the dependency of the binding on MHC concentration in the presence or absence of β_2 microglobulin.

30 Fig. 7 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

Fig. 8 Scatchard Analysis of binding to MHC A11 confirming an apparent K_D of 6nM.

35 Fig. 9 shows the binding of a radioactively labeled peptide of the invention to MHC A1 as measured by % bound reactivity.

Fig. 10 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

Fig. 11 Scatchard Analysis of binding to MHC A1 confirming an apparent K_D of 21nM.

Fig. 12 shows the binding of two peptides of this invention as a function of MHC A24 concentration as measured by % bound reactivity.

Fig. 13 shows the dose dependent inhibition of binding to MHC A24 with the addition of unlabeled peptides.

Figs. 14(a) and 14(b) show the Scatchard Analysis of binding to MHC A24 of the two peptides confirming a K_D of 30 and 60nM, respectively.

Fig. 15 shows the effect on MHC class 1 molecules of β_2 microglobulin and a peptide of choice on acid-stripped PHA blasts.

Fig. 16 shows CTL induction using GC43 A2.1 responders and autologous acid-stripped PBMCs or PHA blasts loaded with the 777.03-924.07-927.32 peptide pool.

Fig. 17 shows CTL induction using X351 or X355 A2.1 responders and autologous acid stripped PBMCs or PHA blasts as stimulators after loading with the 1044.04-1044.05-1044.06 peptide pool.

Fig. 18 shows CTL induction using GC49 A2.1 responders and Autologous Acid stripped PHA blasts as stimulators after loading with 939.03 peptide.

Fig. 19 shows CTL induction using GC66 A1 responders and autologous acid stripped PBMCs as stimulators after loading of peptide 938.01.

Fig. 20 illustrates the lysis of peptide sensitized targets and endogenous targets following stimulation with SAC-I activated PBMCs loaded with a MAGE 3 peptide.

Fig. 21 shows a comparison of the acid strip loading with the cold temperature incubation.

Fig. 22 shows a CTL response to an immunogenic peptide for MAGE/A11.

Fig. 23 shows a CTL response to an immunogenic peptide for HIV/A3.

Fig. 24 shows a CTL response to an immunogenic peptide for HCV/A3.

Fig. 25 shows a CTL response to an immunogenic peptide for HBV/A1.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral
10 diseases or cancers, for which the amino acid sequence of the potential antigen targets is known.

 Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B
15 core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

 Peptides comprising these epitopes are synthesized
20 and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I
25 assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or in
30 vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

 The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell
35 surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles.

The peptide binding motifs of the invention are relatively specific for each allelic subtype.

For peptide-based vaccines, the peptides of the present invention preferably comprise a motif recognized by an MHC I molecule having a wide distribution in the human population. Since the MHC alleles occur at different frequencies within different ethnic groups and races, the choice of target MHC allele may depend upon the target population. Table 1 shows the frequency of various alleles at the HLA-A locus products among different races. For instance, the majority of the Caucasoid population can be covered by peptides which bind to four HLA-A allele subtypes, specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the majority of the Asian population is encompassed with the addition of peptides binding to a fifth allele HLA-A11.2.

TABLE 1

	<u>A Allele/Subtype</u>	<u>N(69)*</u>	<u>A(54)</u>	<u>C(502)</u>
5	A1	10.1(7)	1.8(1)	27.4(138)
	A2.1	11.5(8)	37.0(20)	39.8(199)
	A2.2	10.1(7)	0	3.3(17)
	A2.3	1.4(1)	5.5(3)	0.8(4)
	A2.4	-	-	-
10	A2.5	-	-	-
	A3.1	1.4(1)	0	0.2(0)
	A3.2	5.7(4)	5.5(3)	21.5(108)
	A11.1	0	5.5(3)	0
	A11.2	5.7(4)	31.4(17)	8.7(44)
15	A11.3	0	3.7(2)	0
	A23	4.3(3)	-	3.9(20)
	A24	2.9(2)	27.7(15)	15.3(77)
	A24.2	-	-	-
	A24.3	-	-	-
20	A25	1.4(1)	-	6.9(35)
	A26.1	4.3(3)	9.2(5)	5.9(30)
	A26.2	7.2(5)	-	1.0(5)
	A26V	-	3.7(2)	-
	A28.1	10.1(7)	-	1.6(8)
25	A28.2	1.4(1)	-	7.5(38)
	A29.1	1.4(1)	-	1.4(7)
	A29.2	10.1(7)	1.8(1)	5.3(27)
	A30.1	8.6(6)	-	4.9(25)
	A30.2	1.4(1)	-	0.2(1)
30	A30.3	7.2(5)	-	3.9(20)
	A31	4.3(3)	7.4(4)	6.9(35)
	A32	2.8(2)	-	7.1(36)
	Aw33.1	8.6(6)	-	2.5(13)
	Aw33.2	2.8(2)	16.6(9)	1.2(6)
35	Aw34.1	1.4(1)	-	-
	Aw34.2	14.5(10)	-	0.8(4)
	Aw36	5.9(4)	-	-

40 Table compiled from B. DuPont, Immunobiology of HLA, Vol. I, Histocompatibility Testing 1987, Springer-Verlag, New York 1989.

45 * N - negroid; A = Asian; C = caucasoid. Numbers in parenthesis represent the number of individuals included in the analysis.

50 The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would

assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G.

The procedures used to identify peptides of the present invention generally follow the methods disclosed in Falk et al., Nature 351:290 (1991), which is incorporated herein by reference. Briefly, the methods involve large-scale isolation of MHC class I molecules, typically by immunoprecipitation or affinity chromatography, from the appropriate cell or cell line. Examples of other methods for isolation of the desired MHC molecule equally well known to the artisan include ion exchange chromatography, lectin chromatography, size exclusion, high performance ligand chromatography, and a combination of all of the above techniques.

A large number of cells with defined MHC molecules, particularly MHC Class I molecules, are known and readily available. For example, human EBV-transformed B cell lines have been shown to be excellent sources for the preparative isolation of class I and class II MHC molecules. Well-characterized cell lines are available from private and commercial sources, such as American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A.); National Institute of General Medical Sciences 1990/1991 Catalog of Cell Lines (NIGMS) Human Genetic Mutant Cell Repository, Camden, NJ; and ASHI Repository, Bingham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Table 2 lists some B cell lines suitable for use as sources for HLA-A alleles. All of these cell lines can be grown in large batches and are therefore useful for large scale production of MHC molecules. One of skill will recognize that these are merely exemplary cell lines and that

many other cell sources can be employed. Similar EBV B cell lines homozygous for HLA-B and HLA-C could serve as sources for HLA-B and HLA-C alleles, respectively.

TABLE 2

HUMAN CELL LINES (HLA-A SOURCES)

5		
	HLA-A allele	B cell line
10	A1	MAT COX (9022) STEINLIN (9087)
15	A2.1	JY
20	A3.2	EHM (9080) HO301 (9055)GM3107
25	A24.1	KT3(9107),TISI (9042)
30	A11	BVR (GM6828A) WT100 (GM8602),WT52 (GM8603)
35		

In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used.

For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B, and HLA-C molecules. Several mAb reagents for the isolation of HLA-A molecules are available (Table 3). Thus, for each of the targeted HLA-A alleles, reagents are available that may be used for the direct isolation of the HLA-A molecules. Affinity columns prepared with these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and

one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

TABLE 3

ANTIBODY REAGENTS

anti-HLA	Name
HLA-A1	12/18
HLA-A3	GAPA3 (ATCC, HB122)
HLA-11,24.1	A11.1M (ATCC, HB164)
HLA-A,B,C	W6/32 (ATCC, HB95)
monomorphic	B9.12.1 (INSERM-CNRS)
HLA-B,C	B.1.23.2 (INSERM-CNRS)
monomorphic	

The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class I molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a combination thereof.

Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]). Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecular binding assay as described in Example 10, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S (Melief, et al., Eur. J. Immunol. 21:2963 [1991]).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., Nature, 319:675 (1986); Ljunggren, et

al., Eur. J. Immunol. 21:2963-2970 (1991)), and the human somatic T cell hybridoma, T-2 (Cerundolo, et al., Nature 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce in vitro primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line (see Schneider J. Embryol. Exp. Morphol. 27:353-365 [1927]). That have been transfected with the appropriate human class I MHC allele encoding genes and the human B₂ microglobulin genes.

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100 μ M of peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded antigen-presenting cells are then incubated with the responder cell populations in vitro for 7 to 10 days under optimized culture conditions. Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide sequence was derived.

Specificity and MHC restriction of the CTL is determined by testing against different peptide target cells expressing appropriate or inappropriate human MHC class I. The peptides that test positive in the MHC binding assays and give rise to specific CTL responses are referred to herein as immunogenic peptides.

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or isolated from natural sources such as whole viruses or tumors.

Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles. The polypeptides or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of 9 or 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and

Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

5 The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated
10 that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins,
15 such as L- α -amino acids, or their D-isomers, but may include non-natural amino acids as well, such as β - γ - δ -amino acids, as well as many derivatives of L- α -amino acids.

 Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of
20 electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T
25 cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing
30 necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide.
35 In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 4 when it is desired to finely modulate the characteristics of the peptide.

TABLE 4

<u>Original Residue</u>	<u>Exemplary Substitution</u>
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 4, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al.,

Eur. J. Drug Metab Pharmacokin. 11:291-302 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous trichloroacetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

The peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide desired attributes other than improved serum half life. For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which assists in priming CTL. Lipids have been identified as agents capable of assisting the priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, E. coli lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine (P_3CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., Nature 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P_3CSS , for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with P_3CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support, or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C

terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH₂ acylation, e.g., by alkanoyl (C₁-C₂₀) or thioglycolyl acetylation, terminal-carboxyl amidation, 5 e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, 10 the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, 15 Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), supra.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression 20 vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York 25 (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length 30 contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can 35 then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable

host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression
5 vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable
10 bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for
15 administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma,
20 lymphoma, CMV and condyloma acuminatum.

For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of
25 infection can be treated with the immunogenic peptides separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to
30 cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and
35 severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic

administration) from about 1.0 μ g to about 5000 μ g of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μ g to about 1000 μ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states; that is, life-threatening or potentially life threatening situations.

In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in

the range of about 1.0 μ g to about 5000 μ g, preferably about 5 μ g to 1000 μ g for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a
5 prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

10 The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the
15 invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine,
20 hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution
25 prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium
30 lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about
35 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose,

sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as bovine serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The

vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant.

Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P₃CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 µg to about 5000 µg per 70 kilogram patient, more commonly from about 10 µg to about 500 µg mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods

useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL ex vivo, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell). In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T cells, the culture of stimulator cells is maintained in an appropriate serum-free medium.

Prior to incubation of the stimulator cells with the cells to be activated, e.g., precursor CD8+ cells, an amount of antigenic peptide is added to the stimulator cell culture, of sufficient quantity to become loaded onto the human Class I molecules to be expressed on the surface of the stimulator cells. In the present invention, a sufficient amount of peptide is an amount that will allow about 200, and preferably 200 or more, human Class I MHC molecules loaded with peptide to be expressed on the surface of each stimulator cell. Preferably, the stimulator cells are incubated with >20µg/ml peptide.

Resting or precursor CD8+ cells are then incubated in culture with the appropriate stimulator cells for a time period sufficient to activate the CD8+ cells. Preferably, the CD8+ cells are activated in an antigen-specific manner. The
5 ratio of resting or precursor CD8+ (effector) cells to stimulator cells may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to culturing conditions and the
10 nature and severity of the disease condition or other condition for which the within-described treatment modality is used. Preferably, however, the lymphocyte:stimulator cell ratio is in the range of about 30:1 to 300:1. The
15 effector/stimulator culture may be maintained for as long a time as is necessary to stimulate a therapeutically useable or effective number of CD8+ cells.

The induction of CTL *in vitro* requires the specific recognition of peptides that are bound to allele specific MHC class I molecules on APC. The number of specific MHC/peptide complexes per APC is crucial for the stimulation of CTL,
20 particularly in primary immune responses. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a secondary CTL response, the successful activation of a CTL precursor (pCTL) during primary response requires a significantly higher
25 number of MHC/peptide complexes. Peptide loading of empty major histocompatibility complex molecules on cells allows the induction of primary cytotoxic T lymphocyte responses. Peptide loading of empty major histocompatibility complex molecules on cells enables the induction of primary cytotoxic T lymphocyte
30 responses.

Since mutant cell lines do not exist for every human MHC allele, it is advantageous to use a technique to remove endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the
35 immunogenic peptides of interest. The use of non-transformed (non-tumorigenic), non-infected cells, and preferably, autologous cells of patients as APC is desirable for the design of CTL induction protocols directed towards development

of ex vivo CTL therapies. This application discloses methods for stripping the endogenous MHC-associated peptides from the surface of APC followed by the loading of desired peptides.

5 A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its $\alpha 1$ and $\alpha 2$ domains, and 3) a non-covalently associated non-polymorphic light chain, β_2 microglobulin. Removing the bound peptides and/or dissociating the β_2 microglobulin from the complex 10 renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation. All MHC class I molecules isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step is to remove all endogenous peptides 15 bound to MHC class I molecules on the APC without causing their degradation before exogenous peptides can be added to them.

Two possible ways to free up MHC class I molecules of bound peptides include lowering the culture temperature from 20 37°C to 26°C overnight to destabilize β_2 microglobulin and stripping the endogenous peptides from the cell using a mild acid treatment. The methods release previously bound peptides into the extracellular environment allowing new exogenous peptides to bind to the empty class I molecules. 25 The cold-temperature incubation method enables exogenous peptides to bind efficiently to the MHC complex, but requires an overnight incubation at 26°C which may slow the cell's metabolic rate. It is also likely that cells not actively synthesizing MHC molecules (e.g., resting PBMC) would not 30 produce high amounts of empty surface MHC molecules by the cold temperature procedure.

Harsh acid stripping involves extraction of the peptides with trifluoroacetic acid, pH 2, or acid denaturation of the immunoaffinity purified class I-peptide complexes.

35 These methods are not feasible for CTL induction, since it is important to remove the endogenous peptides while preserving APC viability and an optimal metabolic state which is critical for antigen presentation. Mild acid solutions of pH 3 such as

glycine or citrate-phosphate buffers have been used to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is especially effective, in that only the MHC class I molecules are destabilized (and associated peptides released), while other surface antigens remain intact, including MHC class II molecules. Most importantly, treatment of cells with the mild acid solutions do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of the endogenous peptides occurs in two minutes at 4°C and the APC is ready to perform its function after the appropriate peptides are loaded. The technique is utilized herein to make peptide-specific APCs for the generation of primary antigen-specific CTL. The resulting APC are efficient in inducing peptide-specific CD8+ CTL.

Activated CD8+ cells may be effectively separated from the stimulator cells using one of a variety of known methods. For example, monoclonal antibodies specific for the stimulator cells, for the peptides loaded onto the stimulator cells, or for the CD8+ cells (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged molecules may then be extracted from the stimulator-effector cell admixture via appropriate means, e.g., via well-known immunoprecipitation or immunoassay methods.

Effective, cytotoxic amounts of the activated CD8+ cells can vary between *in vitro* and *in vivo* uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1×10^6 to about 1×10^{12} , more preferably about 1×10^8 to about 1×10^{11} , and even more preferably, about 1×10^9 to about 1×10^{10} activated CD8+ cells are utilized for adult humans, compared to about 5×10^6 - 5×10^7 cells used in mice.

Preferably, as discussed above, the activated CD8+ cells are harvested from the cell culture prior to

administration of the CD8+ cells to the individual being treated. It is important to note, however, that unlike other present and proposed treatment modalities, the present method uses a cell culture system that is not tumorigenic.

5 Therefore, if complete separation of stimulator cells and activated CD8+ cells is not achieved, there is no inherent danger known to be associated with the administration of a small number of stimulator cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

10 Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg. For example, administration of activated CD8+ cells via intravenous
15 infusion is appropriate.

The immunogenic peptides of this invention may also be used to make monoclonal antibodies. Such antibodies may be useful as potential diagnostic or therapeutic agents.

The peptides may also find use as diagnostic
20 reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an
25 affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic infection.

The following examples are offered by way of illustration, not by way of limitation.

30

Example 1

Class I antigen isolation

A flow diagram of an HLA-A antigen purification scheme is presented in Figure 1. Briefly, the cells bearing
35 the appropriate allele were grown in large batches (6-8 liters yielding $\sim 5 \times 10^9$ cells), harvested by centrifugation and washed. All cell lines were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and

antibiotics. For large-scale cultures, cells were grown in roller bottle culture in RPMI 1640 with 10% FBS or with 10% horse serum and antibiotics. Cells were harvested by centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259 rotor and washed three times with phosphate-buffered saline (PBS) (0.01 M PO_4 , 0.154 M NaCl, pH 7.2).

Cells were pelleted and stored at -70°C or treated with detergent lysing solution to prepare detergent lysates. Cell lysates were prepared by the addition of stock detergent solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci. Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0] to the cell pellets (previously counted) at a ratio of 50-100 $\times 10^6$ cells per ml detergent solution. A cocktail of protease inhibitors was added to the premeasured volume of stock detergent solution immediately prior to the addition to the cell pellet. Addition of the protease inhibitor cocktail produced final concentrations of the following: phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5 $\mu\text{g/ml}$; leupeptin, 10 $\mu\text{g/ml}$; pepstatin, 10 $\mu\text{g/ml}$; iodoacetamide, 100 μM ; and EDTA, 3 ng/ml. Cell lysis was allowed to proceed at 4°C for 1 hour with periodic mixing. Routinely 5-10 $\times 10^9$ cells were lysed in 50-100 ml of detergent solution. The lysate was clarified by centrifugation at 15,000 $\times g$ for 30 minutes at 4°C and subsequent passage of the supernatant fraction through a 0.2 μ filter unit (Nalgene).

The HLA-A antigen purification was achieved using affinity columns prepared with mAb-conjugated Sepharose beads. For antibody production, cells were grown in RPMI with 10% FBS in large tissue culture flasks (Corning 25160-225). Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein-A-Sepharose (Sigma). Briefly, saturated ammonium sulfate was added slowly with stirring to the tissue culture supernatant to 45% (volume to volume) overnight at 4°C to precipitate the immunoglobulins. The precipitated proteins were harvested by centrifugation at 10,000 $\times g$ for 30 minutes. The precipitate was then dissolved

in a minimum volume of PBS and transferred to dialysis tubing (Spectro/Por 2, Mol. wt. cutoff 12,000-14,000, Spectrum Medical Ind.). Dialysis was against PBS (≥ 20 times the protein solution volume) with 4-6 changes of dialysis buffer over a 24-48 hour period at 4°C. The dialyzed protein solution was clarified by centrifugation (10,000 x g for 30 minutes) and the pH of the solution adjusted to pH 8.0 with 1N NaOH. Protein-A-Sepharose (Sigma) was hydrated according to the manufacturer's instructions, and a protein-A-Sepharose column was prepared. A column of 10 ml bed volume typically binds 50-100 mg of mouse IgG.

The protein sample was loaded onto the protein-A-Sepharose column using a peristaltic pump for large loading volumes or by gravity for smaller volumes (< 100 ml). The column was washed with several volumes of PBS, and the eluate was monitored at A280 in a spectrophotometer until base line was reached. The bound antibody was eluted using 0.1 M citric acid at suitable pH (adjusted to the appropriate pH with 1N NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base was used to neutralize the eluate. Fractions containing the antibody (monitored by A280) were pooled, dialyzed against PBS and further concentrated using an Amicon Stirred Cell system (Amicon Model 8050 with YM30 membrane). The anti-A2 mAb, BB7.2, and the anti-A3 mAb, GAPA3, are particularly useful for affinity purification.

The HLA-A antigen was purified using affinity columns prepared with mAb-conjugated Sepharose beads. The affinity columns were prepared by incubating protein-A-Sepharose beads (Sigma) with affinity-purified mAb as described above. Five to 10 mg of mAb per ml of bead is the preferred ratio. The mAb bound beads were washed with borate buffer (borate buffer: 100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the washes show A280 at based line. Dimethyl pimelimidate (20 mM) in 200 mM triethanolamine was added to covalently crosslink the bound mAb to the protein-A-Sepharose (Schneider et al., J. Biol. Chem. 257:10766 (1982)). After incubation for 45 minutes at room temperature on a rotator, the excess crosslinking

reagent was removed by washing the beads twice with 10-20 ml of 20 mM ethanolamine, pH 8.2. Between each wash the slurry was placed on a rotator for 5 minutes at room temperature. The beads were washed with borate buffer and with PBS plus
5 0.02% sodium azide.

The cell lysate ($5-10 \times 10^9$ cell equivalents) was then slowly passed over a 5-10 ml affinity column (flow rate of 0.1-0.25 ml per minute) to allow the binding of the antigen to the immobilized antibody. After the lysate was allowed to
10 pass through the column, the column was washed sequentially with 20 column volumes of detergent stock solution plus 0.1% sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0. The HLA-A antigen bound to the mAb was eluted with a basic
15 buffer solution (50 mM diethylamine in water). As an alternative, acid solutions such as 0.15-0.25 M acetic acid were also used to elute the bound antigen. An aliquot of the eluate (1/50) was removed for protein quantification using either a colorimetric assay (BCA assay, Pierce) or by SDS-
20 PAGE, or both. SDS-PAGE analysis was performed as described by Laemmli (Laemmli, U.K., Nature 227:680 (1970)) using known amounts of bovine serum albumin (Sigma) as a protein standard.

Allele specific antibodies were used to purify the specific MHC molecule. In the case of HLA-A2 and HLA-A3 mAbs
25 BB7.2 and GAPA3 were used respectively. An example of SDS PAGE analysis of purified HLA-A3.2 molecules is shown in Figure 2.

Figure 2 shows SDS-PAGE (12.5%) analysis of affinity purified HLA-A3.2 from the cell line EHM. An affinity column
30 (10 ml) was prepared with protein A-sepharose beads coupled to the monoclonal antibody GAPA3 which is specific for HLA-A3. A detergent lysate of 5×10^9 cells was passaged over the column and the column was washed extensively. The bound HLA-A3.2 molecules were eluted from the column with 0.15M acetic acid,
35 50 ml. One ml of the eluate was removed and lyophilized to concentrate the sample. The sample was taken up to 50 μ l with Laemmli sample buffer and 20 μ l were loaded in lane 2. Lane 1 contained molecular weight standards: Myosin, 230 kD; β -

galactosidase, 116kD; phosphorylase B, 97.4kD; bovine serum albumin, 66.2kD; ovalbumin, 45kD; carbonic anhydrase, 31kD; soybean trypsin inhibitor, 21.5kD; and lysozyme, 14.4kD.

Standard concentrations of bovine serum albumin were run in lanes 8, 10 μ g, 9, 3 μ g, and 10, 1 μ g to aid in the estimation of protein yield. For this particular HLA-A3.2 preparation, the estimated yield was approximately 112 μ g.

For HLA-A11, A24.1 and A1, an alternative protocol was used whereby anti-HLA-B and C monoclonal antibodies were used to deplete HLA-B and C molecules. The remaining HLA-A molecules were subsequently purified using the W6/32 mAb as described below.

Based on the density of class I expression as indicated by the results of immunofluorescent staining analysis, it is anticipated that average yields of class I antigen isolated from the EBV B cell lines will range from 800-1200 μ g per 10^{10} cell equivalents.

Example 2

An alternative class I purification protocol

HLA-A2.1 molecules were isolated using the mAb B1.23.2 which detects an epitope expressed by HLA-B and C allele molecules, but not by HLA-A antigens. The mAb, W6/32, detects all human class I molecules, including HLA-A, B and C. As mentioned above, these mAbs react well with the B cell lines serving as sources of HLA-A antigens. The B1.23.2 mAb reacts with the various human B cell lines, but fails to react with a mouse cell line that expresses a transfected HLA-A2.1 protein or a chimeric A2.1 mouse K^b molecule. It does react with the human cell line, CIR (Alexander, J., et al., Immunogenetics, 29, 380 [1989]), that lacks expression of HLA-A and B molecules, but expresses low levels of HLA-C molecules. This pattern of reactivity illustrates how the B1.23.2 mAb can be used to deplete the B cell lysates of HLA-B and C molecules.

Affinity columns were prepared using the affinity-purified B1.23.2 and W6/32 mAbs, respectively, as described above. The procedures for the preparation of the affinity

columns are essentially identical to the procedures described for the preparation of the allele-specific mAb columns described above. The B1.23.2 mAb affinity column was used to deplete the detergent lysates of HLA-B and C molecules using the protocol as described above. The cell lysate depleted of HLA-B and C was then passed over a W6/32 mAb affinity column. The MHC molecule that was eluted from this second passage was the A allele product.

This alternative affinity purification is useful for the purification of any HLA-A allele product, and does not rely on the need for allele-specific mAbs. In addition, it could also be used to isolate any class I molecule type from transfected cell lines.

Example 3

Isolation and sequencing of naturally processed peptides

For the HLA-A preparations derived from the base (50 mM diethylamine) elution protocol, the eluate was immediately neutralized with 1 N acetic acid to pH 7.0-7.5. The neutralized eluate was concentrated to a volume of 1-2 ml in an Amicon stirred cell [Model 8050, with YM3 membranes (Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was added to the concentrator to remove the non-volatile salts, and the sample was concentrated to approximately 1 ml. A small sample (1/50) was removed for protein quantitation as described above. The remainder was recovered into a 15 ml polypropylene conical centrifuge tube (Falcon, 2097) (Becton Dickinson). Glacial acetic acid was added to obtain a final concentration of 10% acetic acid. The acidified sample was placed in a boiling water bath for 5 minutes to allow for the dissociation of the bound peptides. The sample was cooled on ice, returned to the concentrator and the filtrate was collected. Additional aliquots of 10% acetic acid (1-2 ml) were added to the concentrator, and this filtrate was pooled with the original filtrate. Finally, 1-2 ml of distilled water was added to the concentrator, and this filtrate was pooled as well.

The retentate contains the bulk of the HLA-A heavy chain and β_2 -microglobulin, while the filtrate contains the naturally processed bound peptides and other components with molecular weights less than about 3000. The pooled filtrate material was lyophilized in order to concentrate the peptide fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography) separation of the peptide fractions, the lyophilized sample was dissolved in 50 μ l of distilled water, or into 0.1% trifluoroacetic acid (TFA) (Applied Biosystems) in water and injected to a C18 reverse-phase narrow bore column (Beckman C18 Ultrasphere, 10 x 250 mm), using a gradient system described by Stone and Williams (Stone, K.L. and Williams K.R., in, Macromolecular Sequencing and Synthesis; Selected Methods and Applications, A.R. Liss, New York, 1988, pp. 7-24. Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The flow rate was 0.250 ml/minute with the following gradient: 0-60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-98% B. The Gilson narrow bore HPLC configuration is particularly useful for this purpose, although other configurations work equally well.

A large number of peaks were detected by absorbance at 214 nm, many of which appear to be of low abundance (Fig. 3). Whether a given peak represents a single peptide or a peptide mixture was not determined. Pooled fractions were then sequenced to determine motifs specific for each allele as described below.

Pooled peptide fractions, prepared as described above were analyzed by automated Edman sequencing using the Applied Biosystems Model 477A automated sequencer. The sequencing method is based on the technique developed by Pehr Edman in the 1950s for the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids.

The protein or peptide to be sequenced was held by a 12-mm diameter porous glass fiber filter disk in a heated, argon-purged reaction chamber. The filter was generally pre-

treated with BioBrene PlusTM and then cycled through one or more repetitions of the Edman reaction to reduce contaminants and improve the efficiency of subsequent sample sequencing. Following the pre-treatment of the filter, a solution of the sample protein or peptide (10 pmol-5 nmol range) was loaded onto the glass filter and dried. Thus, the sample was left embedded in the film of the pre-treated disk. Covalent attachment of the sample to the filter was usually not necessary because the Edman chemistry utilized relatively apolar solvents, in which proteins and peptides are poorly soluble.

Briefly, the Edman degradation reaction has three steps: coupling, cleavage, and conversion. In coupling step, phenylisothiocyanate (PITC) is added. The PITC reacts quantitatively with the free amino-terminal amino acid of the protein to form the phenylthiocarbamyl-protein in a basic environment. After a period of time for the coupling step, the excess chemicals are extracted and the highly volatile organic acid, trifluoroacetic acid, TFA, is used to cleave the PITC-coupled amino acid residue from the amino terminus of the protein yielding the anilinothiazolinone (ATZ) derivative of the amino acid. The remaining protein/peptide is left with a new amino terminus and is ready for the next Edman cycle. The ATZ amino acid is extracted and transferred to a conversion flask, where upon addition of 25% TFA in water, the ATZ amino acid is converted to the more stable phenylthiohydantoin (PTH) amino acid that can be identified and quantified following automatic injection into the Model 120 PTH Analyzer which uses a microbore C-18 reverse-phase HPLC column for the analysis.

In the present procedures, peptide mixtures were loaded onto the glass filters. Thus, a single amino acid sequence usually does not result. Rather, mixtures of amino acids in different yield are found. When the particular residue is conserved among the peptides being sequenced, increased yield for that amino acid is observed.

Example 4

Definition of an A3.2 specific motif

There is some ambiguity in the international nomenclature of A3 alleles. The A3.2 allele herein is expressed by cell lines EHM, HO301, and GM3107. This particular subtype is currently referred to as the 3.2 allele (Yang, in Immunobiology of HLA, Vol. 1, Dupont ed., Springer-Verlag, New York pp. 43-44 and 54-55, 1989), or the product of the A*0301 gene (its sequence corresponds to the one published by Strachan, et al., EMBO J., 3:887 (1984), and has been verified by direct cloning and sequencing of the A3 gene found in EHM cell line. The HLA-A3.2 encoded by the A*0301 gene referred to in this document is the commonly expressed HLA-A3 allelic form.

In one case using MAT cells, pooled peptide fractions prepared as described in Example 3 above were obtained from HLA-A3.2 homozygous cell lines, for example, CM3107. The pooled fractions were HPLC fractions corresponding to 7% to 19% CH₃CN. For this class I molecule, this region of the chromatogram was most abundant in peptides. Data from independent experiments were averaged as described below.

The amino acid sequence analyses from four independent experiments were analyzed and the results are shown in Table 5. For each position except the first, the data were analyzed by modifying the method described by Falk et al. to allow for comparison of experiments from different HLA types. This modified procedure yielded quantitative yet standardized values while allowing the averaging of data from different experiments involving the same HLA type.

The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation cycle) and 16 columns (each representing one of the twenty amino acids; W, C, R and H were eliminated for technical reasons. The data corresponding to the first row (first cycle) was not considered further because, this cycle is usually heavily contaminated by free amino acids.). The values of each row were summed to yield a total pmoles value for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value, to determine what fraction of the total signal is

attributable to each amino acid at each cycle. By doing so, an "Absolute Frequency" table was generated. This absolute frequency table allows correction for the declining yields of each cycle.

5 Starting from the absolute frequency table, a "relative frequency" table was then generated to allow comparisons among different amino acids. To do so the data from each column was summed, and then averaged. Then, each value was divided next by the average column value to obtain
10 relative frequency values. These values quantitate, in a standardized manner, increases and decreases per cycle, for each of the different sixteen amino acid types. Tables generated from data from different experiments can thus be added together to generate average relative frequency values
15 (and their standard deviations). All standard deviations can then be averaged, to estimate a standard deviation value applicable to the samples from each table. Any particular value exceeding 1.00 by more than two standard deviations is considered to correspond to a significant increase.

20 The results of the foregoing analysis for HLA-A3.2 were as follows: at position 2, a 2.2-fold increase in valine (V) with lesser increases (1.5-1.7) for structurally similar residues leucine (L) and methionine (M). At position 3, tyrosine (Y) and aspartic acid (D) showed increases in
25 frequency. At position 7 isoleucine (I) was increased, and at position 8 asparagine (N) and glutamine (Q) were increased. At positions 9 and 10, lysine (K) was increased more than 2-fold over the expected random yield.

 Cysteine was not modified and thus not detected.
30 PTH-tryptophan coeluted with diphenylurea, and in some experiments, PTH-arginine coeluted with the major derivative of PTH-threonine. Therefore, cysteine and tryptophan are not detectable and arginine is detected only in the absence of threonine.

35 Previously described MHC structures showed instances of critically conserved residues at position 2 (or 3) and at the C terminus (either position 9 or 10). These residues are referred to as "conserved" residues. The modified data

analysis of this invention considered the conserved positions at the N and C terminals.

Thus, the HLA-A3.2 motif should have position two occupied by V, L or M, a length of 9 or 10 amino acids, and a C-terminal position occupied by K.

TABLE 5
Summary
HLA-A3.2 Allele-Specific Motif

Position	Conserved Residues
1	-
2	V,L,M
3	Y,D
4	-
5	-
6	-
7	I
8	Q,N
9	K
10	K

Example 5

Definition of HLA-A1-specific peptide motifs

HLA-A1 molecules were isolated and their naturally processed peptides characterized, as described in Example 3 above. In one case using MAT cells, pooled fractions corresponding to 19% to 50% CH₃CN were used. As in the preceding example, residues showing at any given position except the first position, at least a two standard deviation increase over the random expected yield were identified and shown in Table 6. On the basis of these data, only Serine (S) and Threonine (T) were increased at position two. At position 3, aspartic acid (D) and glutamic acid (E) were elevated and at position 9 and 10 tyrosine (Y) showed a marked increase. Other increases noted were proline (P) at position 4 and leucine (L) at position 7. Therefore, the motifs for HLA-A1 based on these data would have residues at position 2 occupied by S or T, a peptide length of 9 or 10 amino acids and a

C-terminal residue of Y. Alternatively, another motif would comprise a D or E at position 3 together with a C terminal residue of Y.

TABLE 6
Summary
HLA-A1 Allele-Specific Motif

	Position	Conserved Residues
5	1	-
10	2	S, T
	3	D, E
	4	P
	5	-
15	6	-
	7	L
	8	-
	9	Y

Example 6

Definition of HLA-A11 allele-specific peptide motifs

HLA-A11 motifs were defined by amino acid sequence analysis of pooled HPLC fractions, in one case corresponding to 7% to 45% CH₃CN of fractionated peptides eluted from HLA-A11 molecules purified from the cell line BVR. On the basis of the data presented in Table 7, a motif for A11 consists of a conserved residue at position 2 of threonine (T) or valine (V), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of lysine (K). At position 3 increases in methionine (M) and phenylalanine (F) were also seen and at position 8 glutamine (Q) was increased.

TABLE 7
Summary
HLA-A11 Allele-Specific Motif

	Position	Conserved Residues
35	1	-
40	2	T, V
	3	M, F
	4	-

47

	5	-
	6	-
	7	-
	8	Q
5	9	K
	10	K

Example 7Definition of HLA-A24.1 Specific Peptide Motifs

10 HLA-A24.1 allele-specific motifs were defined by amino acid sequence analysis of pooled fractions in one case corresponding to 7% to 19% CH₃CN of HPLC fractionated peptides eluted from HLA-A24.1 molecules purified from the cell line KT3. On the basis of the data presented in Table 8 a motif

15 for HLA-A24.1 consists of a conserved residue at position 2 occupied by tyrosine (Y), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of phenylalanine (F) or leucine (L). Increases were also observed at several other positions: isoleucine (I) and methionine (M) at position 3;

20 aspartic acid (D), glutamic acid (E), glycine (G), lysine (K) and proline (P) at position 4; lysine (K), methionine (M) and asparagine (N) at position 5; valine (V) at position 6; asparagine (N) and valine (V) at position 7; and, alanine (A), glutamic acid (E), lysine (K), glutamine (Q) and serine (S) at

25 position 8.

Table 8.

Summary

HLA-A24.1 Allele-Specific Motif

	Position	Conserved Residues
30	1	-
	2	Y
	3	I, M
35	4	D, E, G, K, P
	5	L, M, N
	6	V
	7	N, V

8	A,E,K,Q,S
9	F,L
10	F,A

5

Example 8Identification of immunogenic peptides

Using the motifs identified above for various MHC class I allele amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of these motifs. Sequences for all of the target antigens were obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs was done using the "FINDPATTERNS" program (Devereux, Haeberli and Smithes (1984). Nucleic Acids Research 12(1); 387-395).

The amino acid sequence or the nucleotide sequence encoding products was obtained from the GenBank database. In the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu), and Melanoma Antigen-1 (MAGE-1), a single sequence exists.

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs were identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr were added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 was derived from 9 viral isolates. Motifs were identified on those regions that had no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates were identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral isolates (10-12 viruses) was obtained

from the Los Alamos National Laboratory database (May 1991 release) and analyzed in order to identify motifs that are constant throughout most viral isolates. Motifs that bear a small degree of variation (one residue, in 2 forms) were also added to the peptide list.

Several motifs for each allele shown below were used to screen several antigens. Protein E6 of human papilloma virus (HPV) type 16 using motifs from all of the alleles disclosed above are shown (Table 9). Protein E7 of HPV type 18 was also searched for motifs from all alleles (Table 9). Melanoma antigens MAGE 1, 2 and 3 were searched for motifs from all alleles (Table 10). The antigen PSA was searched for motifs from all alleles (Table 11). Finally, core and envelope proteins from hepatitis C virus were also searched (Table 12). In the tables and the description of the motifs, the conventional symbol letter for each amino acid was used. The letter "X" represents a wild card character (any amino acid).

The following motifs were screened in the present search:

For HLA-A1 (A*0101):

1	XSXXXXXXY
2	XSXXXXXXY
3	XTXXXXXXY
4	XTXXXXXXY
5	XXDXXXXXXY
6	XXDXXXXXXY
7	XXEXXXXXXY
8	XXEXXXXXXY

For HLA-A3.2 (A*0301)

1	XVXXXXXXXXK
2	XVXXXXXXXXK
3	XLXXXXXXXXK
4	XLXXXXXXXXK
5	XMXXXXXXXXK
6	XMXXXXXXXXK

50

For HLA-A11 (A*1101):

1 XTXXXXXXXK
2 XTXXXXXXXK
3 XVXXXXXXXK
4 XVXXXXXXXK

5

For HLA-A24.1 (A*2401):

1 XYXXXXXXF
2 XYXXXXXXF
3 XYXXXXXXL
4 XYXXXXXXL

10

Peptides with MHC Class I Binding Motifs Table 9

51

AA Position	Sequence	Antigen	HLA molecule
5	30 IHDIILECVY	HPV16.E6	A1
	69 VCDKCLKFY	HPV16.E6	A1
	77 YSKISEYRHY	HPV16.E6	A1
	80 ISEYRHYCY	HPV16.E6	A1
	92 GTTLEQQYNK	HPV16.E6	A11
	93 TTLEQQYNK	HPV16.E6	A11
10	106 LLIRCINCQK	HPV16.E6	A3
	2 HGDTPTLHEY	HPV16.E7	A1
	16 QPETTDLYCY	HPV16.E7	A1
	44 QAEPDRAHY	HPV16.E7	A1
	89 IVCPICSQK	HPV16.E7	A3, A11
	3 RFEDPTRRPY	HPV18.E6	A1
15	4 FEDPTRRPY	HPV18.E6	A1
	25 LQDIEITCVY	HPV18.E6	A1
	41 LTEVFEFAFK	HPV18.E6	A11
	72 YSRIRELRHY	HPV18.E6	A1
	84 SVYGDTLEK	HPV18.E6	A3, A11
	101 LLIRCLRCQK	HPV18.E6	A3
20	59 HTMLCMCKK	HPV18.E7	A11
25			

Human Papilloma Virus 16 and 18 (E6 and E7 Proteins)

Peptides with MHC Class I Binding Motifs Table 10

AA Position	Sequence	Antigen	HLA molecule
5	2 SLEQRS LHCK	MAGE 1	A3
	96 SLFRAVITK	MAGE 1	A3
	96 SLFRAVITKK	MAGE 1	A3
	108 DLVGFLLLK	MAGE 1	A3
	128 MLESVIK NYK	MAGE 1	A3
10	128 MLESVIK NY	MAGE 1	A1
	152 QLVFGIDVK	MAGE 1	A3
	161 EADPTGHSY	MAGE 1	A1
	182 LLGDNQIMPK	MAGE 1	A3
	215 WEELSVMEVY	MAGE 1	A1
15	223 VYDGREHSAY	MAGE 1	A1
	238 LLTQDLVQEK	MAGE 1	A3
	239 LTQDLVQEK	MAGE 1	A11
	239 LTQDLVQEKY	MAGE 1	A1
	240 TQDLVQEKY	MAGE 1	A1
20			

Melanoma Antigen MAGE 1

Peptides with MHC Class I Binding Motifs Table 11

AA Position	Sequence	Antigen	HLA molecule
5	21 IVGGWECEK	PSA	A3, A11
	57 LTAAHCIRNK	PSA	A11
	88 VSHSFPHPHY	PSA	A1
	95 PLYDMSLLK	PSA	A3
	178 DVCAQVHPQK	PSA	A3, A11
10	182 QVHPQKVTK	PSA	A3, A11
	236 PSLYTKVVHY	PSA	A1
	239 YTKVVHYRK	PSA	A11
	241 KVVHYRKWIK	PSA	A3, A11
	242 VVHYRKWIK	PSA	A3, A11
15	Prostate Specific Antigen (PSA)		

Peptides with MHC Class I Binding Motifs Table 12

AA Position	Sequence	Antigen	HLA molecule
5	2 STNPKPQRK	HCV	A11
	14 NTNRRPQDVK	HCV	A11
	43 RLGVRATRK	HCV	A3
	302 VQDCNCSIY	HCV	A1
	556 WMNSTGFTK	HCV	A3
10	605 LTPRCMVDY	HCV	A1
	626 FTIFKIRMY	HCV	A1

Hepatitis C Virus (Consensus Sequence)

15

Example 9Quantitative HLA class I binding assay

To verify that motif-containing peptide sequences are indeed capable of binding to the appropriate class I molecules, specific binding assays were established. HLA-A3.2 molecules were purified from GM3107 EBV cells by affinity chromatography using the GAPA3 mAb (anti-A3) to isolate A3.2. Prior to the step, the lysate was depleted of HLA B and C molecules by repeated passages over a B1.23.2 column (this antibody is B,C specific) generally as described in Example 2, above.

As a radiolabeled probe, the peptide 941.12 (KVFPYALINK), containing an A3.2 motif, was used. This peptide contains the anchor residues V₂ and K₁₀, associated with A3.2-specific binders, described above. A Y residue was inserted at position 5 to allow for radiolodination. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

A dose range of purified A3.2 was incubated with 10 nM of 941.12 at pH 7.0 and 23°C, in presence of a protease inhibitor cocktail (1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 µM pepstatin A, 8 mM EDTA, and 200 µM N α_p-tosyl-L-lysine chloromethyl ketone (TLCK)), in presence of 1 µM purified human β2 microglobulin. After two days, the % bound radioactivity was measured by gel filtration over TSK 2000 columns as previously described for class II peptide binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Geffer, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202, which is incorporated herein by reference. (see, Fig. 4). Good binding (in the 60 to 100% range) was observed for A3.2 concentrations ranging between 35 and 300 nM. 30% binding was observed at 15 nM A3.2.

To minimize A3.2 usage and to increase the sensitivity of the assay, a concentration of 5-10 nM A3.2 was selected for further assays. In the experiment shown in Fig. 5, 7nM A3.2 and an equivalent concentration of radiolabeled 941.12 were incubated using the conditions described above and

in the presence of a dose range of three peptides (HBC 18-27 (924.07), a Prostate Specific Antigen peptide (939.01), and HIV nef 73-82 (940.03)). It was found that peptide 940.03 inhibited strongly, with a 50% inhibitory concentration (IC₅₀) of 22 nM, while a weaker inhibition was observed with peptide 939.01 (IC₅₀ 940 nM). Finally, peptide 924.07 did not show any inhibition up to the 30 μ M level. Thus, it is concluded that peptides 940.03 and 939.01 are high and intermediate affinity binders, respectively, while peptide 924.07 is classified as a low affinity or negative binder.

Throughout this disclosure, results have been expressed in terms of IC₅₀'s. Given the conditions in which the assays are run (i.e., limiting MHC and labeled peptide concentrations), these values approximate K_d values. It should be noted that IC₅₀ values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (e.g., Class I preparation, etc.). For example, excessive concentrations of MHC will increase the apparent measured IC₅₀ of a given ligand.

An alternative way of expressing the binding data, to avoid these uncertainties, is as a relative value to a reference peptide. The reference peptide is included in every assay. As a particular assay becomes more, or less, sensitive, the IC₅₀'s of the peptides tested may change somewhat. However, the binding relative to the reference peptide will not change. For example, in an assay run under conditions such that the IC₅₀ of the reference peptide increases 10-fold, all IC₅₀ values will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder should be based on its IC₅₀, relative to the IC₅₀ of the standard peptide.

If the IC₅₀ of the standard peptide measured in a particular assay is different from that reported in the table, then it should be understood that the threshold values used to determine good, intermediate, weak, and negative binders should be modified by a corresponding factor. For example, if

in an A2.1 binding assay, the IC50 of the A2.1 standard (941.01) were to be measured as 8 nM instead of 5 nM, then a peptide ligand would be called a good binder only if it had an IC50 of less than 80 nM (i.e., $8\text{ nM} \times 0.1$), instead of the usual cut-off value of 50 nM.

The experimental system herein described can be used to test binding of large numbers of synthetic peptides to a variety of different class I specificities. Specific binding assays can be performed as follows.

HLA-A11-specific assay

The cell line BVR was used as a source of HLA. The dependency of the binding on MHC concentration in presence or absence of $\beta_2\text{M}$ are shown in Fig. 6, while Fig. 7 depicts the dose dependency of the inhibition by excess unlabeled ligand. Finally, Fig. 8 shows a Scatchard analysis experiment. Values of apparent K_D of ~ 6 nM and of 10% active receptor were obtained, and were remarkable for their similarity to the values obtained for A2.1 and A3.2. The sequence of the peptide used as a radiolabeled probe (940-06) is AVDLYHFLK.

HLA-A1-specific assay

In this case, the EBV cell line Steinlin was used as a source of purified HLA. The same protocol previously applied to purification of other HLA alleles (i.e., depletion of B, C molecules by a B1.23.2 mAb column, followed by purification of A molecules by means of a W632 mAb column) was utilized. On the basis of the pool sequencing data, consensus peptides were synthesized, directly radiolabeled, and tested for HLA binding using the standard protocol (1 mM $\beta_2\text{M}$, 2 days RT incubation in presence of protease inhibitors). A graph illustrating the relationship between % binding and μM input HLA A1 is shown in Fig. 9. From the data, it was concluded that in analogy with what was observed for HLA A2, 3, and 11, as little as 30 nM are sufficient to obtain $\sim 10\%$ binding. The sequence of the peptide used as a radiolabeled probe (944.02) is YLEPAIAKY. In the next set of experiments, the specificity of the assay established was verified by its inhabitability by excess unlabeled peptide. The IC50% was measured (Fig. 10) as

20 nM. Further Scatchard analysis (Fig. 11) verified that the apparent K_D of the interaction corresponded to 21 nM, with a % of active receptor corresponding to 5.1%.

5

HLA-A24 specific assay

HLA A24 molecules were purified from the KT3 EBV cell line. In this case, two consensus peptides whose sequences were based on the pool sequencing data have been synthesized. Their sequences are: 979-01, AYIDNVYKF and 979.02, AYIDNYNKF. The results of experiments in which the % bound of these two peptides as a function of input MHC was measured are shown in Fig. 12. In both cases, 10-15% binding was obtained with as little as 20-50 nM MHC. Cold inhibition experiments (Fig. 13), limiting MHC concentrations, revealed that the binding was readily inhibitable by excess unlabeled peptide, with an apparent K_D of 30 and 60 nM, respectively. Further Scatchard experiments verified values of 136 nM and 28 nM, respectively. The apparent % of available receptor (active MHC) were 8.3% and 7.4%, respectively (Fig. 9a and b). On the basis of these data, peptide 979.02 was arbitrarily selected as standard label indicator for A24 assays. Furthermore, on the basis of the data herein described, we also conclude that the goal of establishing an A24-specific binding assay has been accomplished. In conclusion, specific assays for the five major HLA alleles have been described.

Example 10

Expansion of HLA A Motifs

Establishing *in vitro* binding assays allows one to readily quantitate *in vitro* the binding capacity of various synthetic peptides to the various alleles of interest (HLA A1, A2, A3, A11, and A24). This allows verification of the correctness of the motifs by means of peptides carrying the various HLA A motifs for their capacity to bind purified HLA molecules. Typically, peptides were synthesized with specific HLA motifs embedded in a neutral backbone composed of only alanine residues. In some cases, a K residue was also introduced within the sequence, with the purpose of increasing

solubility. The use of such "neutral" poly A backbones, as applied to the case of class II molecules, has been described in detail, for example, by Jardetzky et al. (Jardetzky et al., EMBO J. 9(6):1797,1990).

5 For example, in the case of A3.2, a motif has been defined with a hydrophobic residue in position 2 and a positive charge (K) in position 9. Thus, to verify that the presence of these two anchor residues would allow, in the context of a poly A backbone, for A3.2 binding, the poly A
10 analog with the sequence AMAAAAAAK was synthesized (Table 13).

Similarly, other peptides carrying other HLA motifs were also synthesized and tested for HLA binding. It was found that in all cases, the presence of the specific HLA motifs was conducive to binding to the relevant HLA allele,
15 with estimated K_D comprised of between 125 and 2.8 nM. In most cases, the binding was also absolutely specific, in that no binding was detected to irrelevant alleles. Only two exceptions to this general rule were observed. Firstly, A3 and A11 peptides crossreacted extensively with each other,
20 perhaps as could have been expected by the fact that the motifs for these two alleles are remarkably similar. Second, some A1 peptides crossreacted, albeit with much lower affinities, on A11 and A3.2.

To further define the structural requirements for the
25 interaction between peptide epitopes and various class I alleles of interest, analogs of 10 residues in length of some of the 9 residue peptides shown in Table 13 were synthesized (Table 14). These analogs were generated by inserting an additional Ala residue within the poly A backbone, so that the
30 anchor residues are not located in positions 2 and 10 (as opposed to 2 and 9 in the previous Table). The results obtained illustrate that motifs of 10 residues are also capable of specifically binding to the relevant class I alleles, albeit with a slightly lower efficiency.

35 In summary, these data confirm that both 9-mer and 10-mer peptides which contain the appropriate motifs can bind HLA. On the basis of these data, 8-mer or 11-mer peptides

should also be capable of binding, even if perhaps with lower affinities.

The data described above show that the presence of certain residues in the anchor positions does allow (at least in a "neutral" poly A backbone) for HLA binding. To investigate to what degree other amino acids (for example, chemically related amino acids) might be tolerated in these crucial anchor positions, analogs of some of the poly A peptides from Table 13 were synthesized, in which the residue present in position 2 (or 3) or 9 was varied. The results of this analysis are shown in Tables 15-19.

In the case of A3.2 (Table 15), in position 2, L, M, I, V, S, A, T, and F were found to be preferred (binding ≥ 0.1 relative to previously defined anchor residues), while C, G, and D were permitted (binding ≥ 0.01 to 0.1 relative to previously defined anchor residues). The substitution of E, because of its similarity to D, in this position should also be tolerated. In position 9, K, R, and Y were preferred. Because of a similarity in nature, that H and F should also be preferred. No other residue was tolerated in position 9 for A3 binding.

In the case of A11 (Table 16), the preferred residues in position 2 were L, M, I, V, A, S, T, G, N (L and Q by similarity). Tolerated were C, F, D (and E by similarity). In position 9, K was preferred and R was tolerated. H should also be tolerated by similarity.

In the case of A24 (Table 17), Y and F were preferred in position 2 (and W by similarity); no other residue was tolerated. In position 9, F, I, and L were preferred (and W and M by extension). No other residue was tolerated.

In the case of A1, three different anchor residues had previously been defined. The results shown in the preceding section show that they act independently of each other (i.e., that two out of three anchors would be sufficient for binding). This is indeed the case. For this reason, analogs containing two anchors were synthesized to define what residues might be preferred or tolerated in each position. The data shown in Table 18 show that in position 2, T, S, and

M are preferred, and no other residue is tolerated. In position 3 (Table 19), D and E are preferred, and A, S (and T by similarity) are tolerated. Finally, in position 9, only Y is preferred, and no other residue appears to be tolerated (Table 19).

Thus, on the basis of the data, it is concluded that peptides carrying any combination of two preferred residues can bind. Peptides containing "imperfect" motifs, i.e., carrying a preferred residue at one position and a tolerated one at the other anchor position, should also be capable of binding, even if with somewhat lower affinity. Using the motifs of this invention for various MHC class I alleles amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of motifs. The results of this motif analysis is shown in Table 23 a - k.

Example 11

Validation of HLA Peptide Binding Motifs with an Unbiased Set of HPV 16 Peptides.

Human Papillomaviruses (HPVs) are implicated in the etiology of cervical cancer (Pfister, H. (1974) *Biology and biochemistry of papillomaviruses*, Rev. Physiol. Biochem. 99:111; zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology*. 184:9) and in up to 10% of total mortality due to cancer worldwide (zur Hausen, H. (1991). *Viruses in Human Cancers*. Science, 254:1167). Cervical cancer is the second most common cause of cancer-related death in females worldwide (Parkin, D. M., Laara, E., and Muir, C. S. (1988), Estimates of the worldwide frequency of sixteen major cancers in (1980). *Int. J. Cancer*. 41:184). HPV DNA is present in more than 90% of the cervical carcinomas and predominantly of the HPV 16 genotype (Resnick, R. M., Cornelissen, M. T., Wright, D. K., Eichinger, G. H., Fox, H. S., ter Schegget, J., and Manos, M. M. (1990). Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J. Natl. Cancer Inst*; Van den Brule, A. J. C., Walboomers, J. M. M., du Maine, M., Kenemans, P., and Meijer,

C. J. L. M. (1991). Difference in prevalence of human papillomavirus genotypes in cytologically normal smears is associated with a history of cervical intraepithelial neoplasia. *Int. J. Cancer.* 48:404). The ability of HPV 16 early region 6 and 7 (E6, E7) open reading frames to in vitro immortalize rodent cells (Yasumoto, S., Burkhardt, A.L., Doniger, J., and DiPaolo, J.A. (1986). Human Papillomaviruses type 16 DNA induced malignant transformation of NIH3T3 cells. *J. Virol.* 57:572) and human keratinocytes (Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J.A. (1987). Trans-formation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.* 61:1061) and to transform human fibroblasts (Smits, H. L., Raadsheer, E., Rood, I., Mehendale, S., Slater, R. M., van der Noordaa, J., and ter Schegget, J. (1988). Induction of anchorage-independent growth of human embryonic fibroblasts with a deletion in the short arm of chromosome 11. *J. Virol.* 62:4538) suggests direct involvement of HPV 16 in the multi-step process of cervical carcinogenesis,

In general T cell immunity, in particular mediated by cytotoxic T lymphocytes (CTL) is important in the defense against virus-induced tumors (Melief, C. J. (1992). Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. *Adv. Cancer Res.* 58:143; Melief, C. J., and Kast, W. M. (1992). Lessons from T cell responses to virus induced tumors for cancer eradication in general. *Cancer Surv.* 13:81). Recently in a mouse model, it was reported that some degree of protection against HPV 16 E7 expressing tumors can be obtained with CTL after immunization with HPV 16 E7 expressing cells (Chen, L., Thomas, E. K., Hu, S. L., Hellström, I., and Hellstrom, K. E. (1991). Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. *Proc. Natl. Acad. Sci.* 88:110; Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellström, K. E., Ledbetter, J. A., McGowan, P., and Linsley, P. S. (1992). Costimulation of Antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell.* 71:1093). In vivo protection by CTL was recently shown in mouse models in which

synthetic peptides containing CTL epitopes were used for efficient priming of mice against virus infections (Schulz, M., Zinkernagel, R. M., and Hengartner, H. (1991).

Peptide-induced antiviral protection by cytotoxic T cells.

5 *Proc. Natl. Acad. Sci. USA* 88:991; Kast, W. M., Roux, L., Curren, J., Blom, H. J. J., Voordouw, A. C., Meleon, R. H., Kolakofski, D., and Melief, C. J. M. (1991). Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with an unbound
10 peptide. *Proc. Natl. Acad. Sci. USA* 88:2283). Moreover in a mouse model it has now been shown that complete protection against HPV 16 induced tumors can be achieved by peptide vaccination with a CTL epitope derived from the viral oncogene E7().

15 The HPV 16 E6 and E7 gene products are the most desirable target antigens for vaccination against HPV 16 induced tumors. Both are retained and highly expressed in HPV 16-transformed cancer cells in vivo (Baker, C. J., Phelps, W. C., Lindgren, V., Braun, M. J., Gonda, M. A., and Howley, P. M. [1987]. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell
20 lines. *J. Virol.* 61:962; Smotkin, D., and Wettstein, F. O. [1986]. Transcription of human papillomavirus type 16 early genes in a cervical cancer and cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA*.
25 83:4680) and involved in the induction and maintenance of cellular transformation in vitro (Crook, T., Morgenstern, J. P., Crawford, L., and Banks, L. [1989]. Continued expression of HPV-16 E7 protein is required for maintenance of the
30 transformed phenotype of cells co-transformed by HPV-16 plus EJ ras. *EMBO J.* 8:513; Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R., and Schiller, J. T. [1989]. HPV 16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* 8:3905). Dependence of in vitro growth
35 of cell lines derived from cervical cancers on the expression of E6 and E7 emphasizes involvement of these oncogenes in maintenance of the phenotype of cervical carcinoma cell lines (Von Knebel Doeberitz, M., Bauknect, T., Bartch, D., and zur

Hausen, H. [1991]. Influence of chromosomal integration on glucocorticoid-regulated transcription of growth-stimulation papillomavirus genes E6 and E7 in cervical carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 88:1411). To determine the CTL epitopes and potential vaccine candidates of HPV 16 for humans, we screened peptides spanning the HPV 16 E6 and E7 protein sequences for their ability to bind to the most frequent human MHC molecules, namely HLA-A1, A3.2, A11.2 and A24. Combined these five alleles will cover about 90% of the world population (Dupont, B., ed. [1987]. *Immunology of HLA Vol. I -- Histocompatibility Testing.* Springer-Verlag, New York).

A complete set of 240 overlapping synthetic peptides of 9 aa length and 8 aa overlap covering the entire HPV 16 E6 and E7 oncogene sequences were synthesized. The peptides were tested for their ability to bind the aforementioned HLA molecules in the binding assay described above. The results of this analysis show the relative affinity of all peptides for the respective HLA alleles and reveal the possible candidate CTL epitopes for use in peptide based vaccines for humans in Tables 20(a)-(d).

The results confirm that peptide binding motif described in this invention for the aforementioned HLA alleles predict which peptide of a protein is likely to bind into the groove of a specified HLA molecule. Since we used a large and unbiased set of peptides, the results of the peptide binding analyses were used to evaluate the value of these motifs both for their predictive capacities and the necessity to have particular anchor aa residues on positions 2, (3) and 9 in a peptide.

Peptides. Peptides were generated by solid phase strategies on a multiple peptide synthesizer (Abimed AMS 422) by repeated cycles in which addition of Fmoc protected amino acids to a resin of polystyrene was alternated with a Fmoc-deprotection procedure (Gausepohl, H., Kraft, M., Boulin, Ch., and Frank, R. W. [1990]. Automated multiple peptide synthesis with BOP activation. in *Proc. of the 11th American peptide symposium.* J. E. Rivier and G. R. Marshall, Ed.

ESCOM, Leiden. 1003-1004). The peptides all carrying a COOH group at the C-terminal end, were cleaved from the resin and side chain protective groups were removed by treatment with aqueous TFA. Peptides were analyzed by reversed phase HPLC lyophilized and dissolved at a concentration of 1 mg/ml in phosphate-buffered saline with 3% DMSO (Sigma, St. Louis, MO 63175) before use. Once dissolved, the peptides were stored at -70° C. Since cysteine containing peptides are susceptible to (air) oxidation during synthesis and handling, these peptides were synthesized with an alanine instead of a cysteine.

Identification of peptides from HPV 16 E6 and E7 proteins that bind to different HLA-A alleles. A complete set of 240 peptides of 9 aa in length and overlapping by 8 aa, covering the sequences of the entire HPV 16 E6 and E7]proteins, was tested for binding to 5 different HLA-A molecules.

The results of this analysis are depicted in Tables 20(a)-(d). Table 20(a) describes the peptides of HPV 16 that bound to HLA-AL molecules. All peptides were tested. Listed are only peptides yielding ratio values of ≥ 0.001 . It can be seen that 2 peptides bound with high affinity to this molecule (>0.1), 6 with intermediate affinity (0.1-0.01) and 1 with low affinity (0.01-0.001). Peptides were ranked by ratio value to allow comparison of data obtained in different experiments. To calculate the concentration of a peptide necessary to yield a 50% inhibition dose (IC_{50}) one has to divide the value of the standard IC_{50} by the ratio. For example, peptide E6-80 has an IC_{50} of 23 nM (81/3.5).

Table 20(b) describes the peptides that bound to HLA-A3.2 molecules. Seven peptides were identified as high affinity binders, 6 as intermediate affinity binders and 13 as low affinity binders. Table 20(c) describes the peptides that bound to HLA-A11.2 molecules. Six high affinity peptides were identified, 4 intermediate affinity binders and 10 low affinity binders. Two high affinity binding peptides (E6-59 IVYRDGNPY and E6-80 ISEYRHYAY) and two weak affinity binding peptides with a Y at the 9th position (E6-42 QQLLRREVY, E6-69

VADKALKFY) were identified for HLA-A11.2. Considering the high binding strength of the first two peptides and the similarity between the HLA-A11.2 motif and the HLA-A3.2 motif in which Y's are preferred at the 9th aa position, tyrosines should be included at the 9th position in the HLA-A11.2 motif. Comparing Tables 21(b) and (c) it is clear that there is a large overlap of peptides that bound to both A3.2 and A11.2 molecules. Eighteen out of 28 E6 and E7 peptides binding to these two HLA molecules overlapped and only 8 peptides were unique for HLA-A3.2 and 2 peptides unique for HLA-A11.2.

Finally, Table 20(d) describes the peptides that bound to HLA-A24 molecules. Here 2 peptides were identified as high affinity binding peptides, 5 as intermediate affinity binding peptides and 5 as low binding peptides. One high affinity peptide (E6-72 KALKFYSKI) and one intermediate affinity peptide (E7-49 RAHYNIVTF) were identified, indicating that an A at the second position should be allowed in the HLA-A24 motif. All these inclusions are indicated in Table 20-e. In analyzing these tables it can be concluded that between 2 and 7 high affinity binding peptides were identified for all of the tested HLA-A molecules. Occasionally some peptides were binding to more alleles. Three peptides (E6-7, E6-37 and E6-79), bound to HLA-A2.1, A3.2 and A11.2. One peptide (E6-38) bound to HLA-A3.2, A11.2 and A24 and two peptides (E6-69 and E6-80) bound to HLA-A1, A3.2 and A11.2. But these crossreactive peptides bound only weakly to one or more of the different HLA molecules. In general, however, it can be concluded that, except for HLA-A3.2 and HLA-A11.2 molecules, almost all HLA molecules bind unique peptides.

Validation of HLA-A peptide binding motifs with an unbiased set of HPV 16 E6 and E7 peptides.

We analyzed how well the motifs for anchor positions described in this invention predicted the binding of a peptide, and also the reverse: how well binding peptides followed the identified motifs. For this, peptides were ranked as high binders, intermediate binders, weak binders, and negative binders and for each peptide the motif prediction based on the anchor motif rules of Table 6 were analyzed. The

overall efficiency of the 2, (3), and 9 anchor motifs was then calculated and this is summarized in Table 20(e). It can be concluded that the motif described above for the different HLA-A molecules are quite accurate. One hundred percent of the HLA-A1, A3.2, and A24 high binders would be predicted as well as 67% of the HLA-11.2. Even for the intermediate binders between 40 and 100% would be predicted depending on the HLA-A molecule analyzed. Furthermore, the percent of weak binding peptides that would be predicted is low and the percent of those peptides that were predicted to bind but actually did not bind is very low for all these alleles.

Analyzed differently, of the 12 peptides predicted to bind to HLA-A1 actually 5 bound with high or intermediate affinity. This indicates that only a few peptides would have to be made to find these potential CTL epitopes. The figures for HLA-A3.2, A11.2, and A24 were 10/32, 7/26, and 4/7, respectively. This implies that the predictive value for all of these alleles is good. Besides a small number of peptides that had not been predicted by the recently described motifs, the (-) in Tables 21(a) - (d), a number of peptides that were predicted by the 2, (3) and 9 anchor motifs did not bind, indicating that having the right anchor residues is not always sufficient for binding and implicating that non-anchor residues can make negative contributions to the binding of a peptide.

Example 12

Presence of a Motif is Necessary But Not Sufficient for High Affinity Class I Binding

To investigate further how the presence of different motifs might influence the capacity of different peptides to bind to the relevant HLA alleles, the sequences of various potential target molecules were scanned for the presence of motif-containing peptides. The peptides thus identified were synthesized and tested for binding. It was found (Table 20) that in the case of A3.2, only 39 (19%) of the 205 peptides bound with high affinity in the 1 to 50 nM range. 22.4% of them bound with intermediate affinities (in the 50 to 500 nM

range), while 34.6% bound weakly (in the 500 nM to 50 μ M range). Finally, 23.9% of them did not bind at all, at least up to the 50 μ M level. In the case of A11, 33 (33%) of the 100 peptides bound with high affinity in the 1 to 50 nM range. 5 35% of them bound with intermediate affinities (in the 50 nM range.), while 24% bound weakly (in the 500 nM to 50 μ M range). Finally, 8% of them did not bind at all, at least up to the 50 μ M level.

Similar results were also obtained (data not shown) 10 in the case of A1 and A24.

The same type of analysis were also performed in the case of 10-mer peptides carrying either the A3.2, and A11 motifs (Tables 22(a) and (b)). It was found that in these cases, the frequency of good binders was even lower (17.5%, 15 and 29.8%, respectively). These data confirm the fact that motif-containing 10-mer peptides can indeed bind, albeit with, in general, reduced affinity.

In summary, the data shown in this section clearly show that the presence of the correct anchor residues is not 20 sufficient *per se* to allow for good HLA binding. It is thus apparent that the nature of the residues contained in positions other than 2(3) and 9 (or 10) can influence binding. The most likely explanation of this observation is that the presence of certain residues (in positions other than 2 and 9) 25 can negate or increase the binding potential of a peptide determinant.

The data shown in the preceding sections describe how specific binding assays can be used to identify, within motif-containing peptides, peptides that are immunogenic. We also 30 wanted to devise an alternative strategy, namely to derive procedures that would be able to predict, within motif-containing peptides, which peptides might be good or intermediate binders and thereby might be immunogenic. In other experiments not shown intermediate or good binders have 35 been shown to be immunogenic. In particular, to identify residues that have a negative impact on binding an analysis of all positions for A3.2, A11, and all motif-containing peptides, both 9-mers and 10-mers is carried out. In the case

of A11, because of the small occurrence of nonbinding peptides, a different cutoff was used such that the analysis compares good and intermediate binders on the one hand to weak and nonbinders on the other.

5

Example 13

Algorithms to Identify Immunogenic Peptides

In light of results presented in Example 13 above, algorithms are developed to provide a more exact predictor of binding based upon the effects of different residues at each position of a peptide sequence, in addition to the anchor or conserved residues. More specifically, we utilize the data bank obtained during the screening of our collection of A1, 3,11 or 24 motif containing peptides to develop an algorithm for each particular allele which assigns a score for each amino acid at each position along a peptide. The score for each residue is taken as the ratio of the frequency of that residue in good and intermediate binders to the frequency of occurrence of that residue in non-binders.

In the present algorithm residues have been grouped by similarity. This avoids the problem encountered with some rare residues, such as tryptophan, where there are too few occurrences to obtain a statistically significant ratio. A listing is made of scores obtained by grouping for each of the twenty amino acids by position for 9-mer peptides containing conserved residues that define their motif (2/9 motifs). A peptide is scored in the algorithm as a product of the scores of each of its residues.

The power of an algorithm to correlate with binding is further underlined by its ability to predict a population of peptides with the highest occurrence of good binders. If one were to rely, for example, solely on the 2/9 motif for predicting 9-mer peptides which bind to a specific MHC allele the large number of peptides containing the motif would be predicted to be good binders. In fact only a relatively small percentage of these peptides are good binders and a somewhat larger percentage are intermediate binders, while a still larger percentage of the peptides predicted by the motif are

either weak or non-binding peptides. In contrast, using the grouped algorithm of this invention a population of peptides are created with a greater percentage of good binders, a still greater percentage of intermediate binders, and a smaller percentage, relative to that predicted by motif-containing peptides, are weak and non-binders.

The present example of an algorithm uses the ratio of the frequency of occurrence of an amino acid in binders and non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill in the art that there are alternative ways of creating a similar algorithm. For example, one could use average binding affinity values, or relative binding of single amino acid substitutions in a motif containing peptide with a poly-alanine backbone to generate an algorithm table.

An algorithm using average binding affinity has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler group ratio algorithm. We create such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of motif containing peptides. The algorithm score for a peptide is then taken as the sum of the scores by position for each of its residues.

Example 14

Preparation of effective HLA allele-specific antigen presenting cells.

This example demonstrates the use of cold temperature incubation or acid stripping/peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC). The APC were used to sensitize precursor cytotoxic T lymphocytes which led to the development of antigen-specific cytotoxic cells. This was accomplished using either phytohemagglutinin (PHA) T-cell blasts or peripheral blood mononuclear cells (PBMC) or staphylococcus aureus Cowan I (SAC-I) activated PBMC as APC. The results are applicable to other APC and to the other MHC alleles.

The following describes sources for materials used in the following examples:

L-Ascorbic acid, Cat #B582, J.T. Baker, Phillipsburg, NJ.

5 Anti-HLA A2 (BB7.2), Cat #HB82, ATCC, Rockville, MD.
Anti-HLA DR (LB3.1), from J. Gorga, Children's Hospital, Pittsburgh, PA.

Anti-HLA Alpha chain pan ABC (9.12.1), from R. DeMars, University of Wisconsin, Madison, WI.

10 Anti-mouse IgG FITC conjugate, Cat #F2883, Sigma, St. Louis, MO.

β_2 microglobulin, Cat #MO114, Scripps Labs, San Diego, CA.

BSA Fraction V, Cat #A9418, Sigma, St. Louis, MO.

15 50cc conical centrifuge tubes, Cat #2070, Falcon, Lincoln, Park, NJ.

Cryo 1°C freezing container, Cat #5100-0001, Nalge, Rochester, NY.

Cryovial, Cat #5000-0012, Nalge, Rochester, NY.

20 Dimethyl sulfoxide (DMSO), Cat #D2650, Sigma, St. Louis, MO.

DNase, Cat #260912, Calbiochem, San Diego, CA.

Dynabeads M-450 goat anti-mouse IgG, Cat #110.06, Dynal, Great Neck, NY.

25 EDTA tetrasodium salt, Cat #ED4SS, Sigma, St. Louis, MO.

FACScan, Becton Dickinson, San Jose, CA.

Fetal calf serum (FCS), Cat #3000, Irvine Scientific, Irvine, CA.

30 Ficoll-Paque, Cat #17-0840-03, Pharmacia, Piscataway, NJ.

Gentamicin, Cat #600-5750AD, Gibco, Grand Island, NY.

L-Glutamine, Cat #9317, Irvine Scientific, Irvine, CA.

35 GS-6KR centrifuge, Beckman Instruments, Palo Alto, CA.

Human AB serum (HS), Cat #100-112, Gemini Bioproducts, Calabasas, CA.

Human rIL-2, Sandoz, Basel, Switzerland.

Human rIL-7, Cat #F1-1587-1, Genzyme, Cambridge, MA.

Isopropanol, Cat #A464-4, Fisher Scientific,
Pittsburgh, PA.

5 MicroCELLector T-150 culture flask for selection of
CD4+ cells, Cat #8030, Applied Immune Sciences, Menlo
Park, CA.

Micromedic automatic gamma counter, ICN Micromedics
Systems, Huntsville, AL.

10 OKT4 hybridoma supernatant, Cat #CRL 8002, ATCC,
Rockville, MD.

Paraformaldehyde, Cat #T-353, Fisher, Pittsburgh, PA.
PBS calcium and magnesium free (CMF), Cat #17-516B,
BioWhittaker, Walkersville, MD.

15 Peptides used in this study were synthesized at Cytel
and described in Table 24 a.

Phytohemagglutinin (PHA), Cat #HA-16, Wellcome,
Dartford, England.

20 RPMI 1640 + Hepes + glutamine, Cat #12-115B,
BioWhittaker, Walkersville, MD.

RPMI 1640 + Hepes + glutamine, Cat #380-2400AJ,
Gibco, Grand Island, NY.

Sodium chloride (NaCl), Cat #3624-05, J.T. Baker,
Phillipsburg, NJ.

25 Sodium (⁵¹Cr) chromate, Cat #NEZ 030, NEN,
Wilmington, DE.

Sodium phosphate monobasic, Cat #S9638, Sigma, St.
Louis, MO.

Triton X-100, Cat #X-100, Sigma, St. Louis, MO.

30 24 well tissue culture plate, Cat #3047, Falcon,
Becton Dickinson, San Jose, CA.

96 well U-bottomed cluster plate, Cat #3799, Costar,
Cambridge, MA.

35 *Culture Medium.* PHA blasts and CTL inductions were done in
RPMI 1640 + Hepes + glutamine (Gibco) supplemented with 2 mM
L-glutamine (Irvine Scientific), 50 µg/ml gentamicin (Gibco),
and 5% heat inactivated pooled human Type AB serum (Gemini

Bioproducts) [RPMI/5% HS]. EBV transformed lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 + Hepes + glutamine (BioWhittaker) supplemented with L-glutamine and gentamicin as above and 10% heat inactivated fetal calf serum (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays were performed in RPMI/10% FCS.

Cytokines. Recombinant human interleukin-2 (rIL-2) (Sandoz) was used at a final concentration of 10 U/ml. Recombinant human interleukin-7 (rIL-7) (Genzyme) was used at a final concentration of 10 ng/ml.

Isolation of Peripheral Blood Mononuclear Cells (PBMC). Whole blood was collected in heparin (10 U/ml) containing syringes and spun in 50cc conical centrifuge tubes (Falcon) at 1600 rpm (Beckman GS-6KR) 15 min. The plasma layer was then removed and 10 ml of the buffy coat collected with a 10 ml pipette using a circular motion. The buffy coat was mixed thoroughly and diluted with an equal volume of serum free RPMI 1640. The diluted buffy coat was then layered over 20 ml Ficoll-Paque (Pharmacia) in a 50cc conical tube and centrifuged 400 x g for 20 min at room temperature with the brake off. The Ficoll-plasma interface containing the PBMCs was collected using a transfer pipet (two interfaces per 50cc tube) and washed three times with 50 ml RPMI (1700, 1500, and 1300 rpm for 10 min.

Freezing and Thawing PBMC. PBMC were frozen at 30×10^6 cells/ml of 90% FCS + 10% DMSO (Sigma), in 1 ml aliquots using cryovials (Nalge). Cryovials were placed in Cryo 1°C freezing containers (Nalge) containing isopropanol (Fisher) and placed at -70°C from 4 hr (minimum) to overnight (maximum). Isopropanol was changed after every 5 uses. Cryovials were transferred to liquid nitrogen for long term storage. PBMC were thawed by continuous shaking in a 37°C water bath until the last crystal was nearly thawed. Cells were immediately diluted into serum free RPMI medium containing DNase 30 µg/ml (to avoid clumping) (Calbiochem), and washed twice.

Depletion of Lymphocyte Subpopulations. CD4 lymphocyte depletion was performed using antibody-coated flasks:

MicroCELLector T-150 flasks for the selection of CD4+ cells (Applied Immune Sciences) were washed according to the
5 manufacturer's instructions with 25 ml PBS CMF + 1 mM EDTA (Sigma) by swirling flasks for 30 sec followed by incubation for 1 hr at room temperature on a flat surface. Buffer was aspirated and flasks were washed 2 additional times by shaking the flasks for 30 sec and maintaining coverage of the binding
10 surface. To each washed flask, 25 ml culture medium + 5% HS were added and incubated for 20 min at room temperature on a flat surface. Media was left in the flask until it was ready to receive the cells. PBMC were thawed in RPMI/5% HS containing 30 µg/ml DNase, and washed twice. HS in the wash
15 blocks Fc receptors on PBMCs. For one flask a maximum of 12×10^7 cells were resuspended in 25 ml culture medium. Culture medium was aspirated from the flask and then the cell suspension was gently added to the MicroCELLector. Flasks containing the cells were incubated for 1 hr at room
20 temperature on a flat surface. At the end of the incubation, the flask was gently rocked from side to side for 10 sec to resuspend the nonadherent cells. Nonadherent CD4 depleted cells were harvested, and then flasks were washed twice with PBS CMF to collect the nonadherent cells. Harvested
25 CD4-depleted cells were pelleted by centrifugation and resuspended in complete culture medium (RPMI/5%/HS).

Generation of PHA Blasts. PBMC were isolated using the standard Ficoll-Paque protocol. Frozen cells were washed
30 twice before use. Cells were cultured at 2×10^6 /ml in RPMI/5% HS containing 1 µg/ml PHA (Wellcome) and 10 U/ml rIL-2. PHA blasts were maintained in culture medium containing 10 U/ml r IL-2 with feeding and splitting as needed. PHA blasts were used as APC on day 6 of culture.

35 Generation of empty class I molecules and peptide loading were only performed by the acid strip method when using these APC.
Acid Stripping/Peptide Loading of PBMC and PHA Blasts. PBMC were isolated using the Ficoll-Paque protocol. When using

frozen cells, PBMC were washed twice before using. PHA blasts were prepared as previously described and washed twice before using. Once cells were prepared, they were washed once in cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc
5 conical centrifuge tube, the cells were resuspended at 10^7 /ml in cold sterile citrate-phosphate buffer [0-13 M L-ascorbic acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma) pH 3, 1% BSA, 3 μ g/ml β_2 microglobulin (Scripps Labs)] and incubated for 2 min on ice. Immediately, 5 volumes of cold
10 sterile neutralizing buffer #1 [0.15 M sodium phosphate monobasic pH 7.5, 1% BSA, 3 μ g/ml β_2 microglobulin, 10 μ g/ml peptide] were added, and the cells were pelleted at 1500 rpm, 5 min at 4°C. Cells were resuspended in 1 volume cold sterile neutralizing buffer #2 [PBS CMF, 1% BSA, 30 lig/ml DNase, 3
15 μ g/ml β_2 microglobulin, 40 μ g/ml peptide] and incubated for 4 hrs at 20°C. Cells were diluted with culture medium to approximately 5×10^6 /ml and irradiated with 6000 rads. Cells were then centrifuged at 1500 rpm for 5 min at room temperature and resuspended in culture medium. The acid
20 stripped/peptide loaded cells were used immediately in the CTL induction cultures (below).

Induction of Primary CTL using Acid Stripped/Peptide Loaded Autologous PBMCs or PHA Blasts as Stimulators. Acid
25 stripping/peptide loading of PBMC and PHA blasts are described above. During the last 4 hr incubation of stimulator cells with peptide, the responder cell population was prepared: Responders were PBMC that were depleted of CD4+ cells (described above). Responder cells were resuspended in
30 culture medium at 3×10^6 /ml. 1 ml of the responder cell suspension was dispensed into each well of a 24-well tissue culture plate (Falcon, Becton Dickinson). The plates were placed in the incubator at 37°C, 5% CO₂ until the stimulator population was ready. Once irradiated, stimulator APC were
35 resuspended in culture medium containing 20 ng/ml rIL-7 at 10^6 /ml for the PBMC, or at 3×10^5 /ml for the PHA blasts. 1 ml of stimulator cell suspension was added per well to the plates containing the responders. On day 7 after induction, a

100 μ l culture medium containing 200 ng/ml rIL-7 was added to each well (20 ng/well rIL-7 final). On day 10 after induction, 100 μ l of culture medium containing 200 U/ml rIL-2 was added to each well (20 U/well rIL-2 final).

5

Antigen Restimulation of CTL. On day 12-14 after the induction, the primary CTL were restimulated with peptide using adherent APC. Autologous PBMC were thawed and washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium at 4×10^6 /ml. 1 ml of cell suspension was added to each well of a 24-well tissue culture plate, and incubated for 2 hrs at 37°C, 5% CO₂. Non-adherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μ g/ml β_2 microglobulin and 20 μ g/ml total peptide was added to each well. APC were incubated for 2 hrs at 37°C, under 5% CO₂ with the peptide and β_2 microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5×10^6 /ml in culture medium was added to each well. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well.

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Cytotoxicity Chromium Release Assay. Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.

25

a. Effector Cell Preparation: the responders, which at this stage are renamed "effectors", were centrifuged and resuspended at 10^7 /ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μ l/well on 96 well U-bottomed cluster plates (Costar), in duplicate.

30

b. Target Cell Preparation: Approximately 16-20 hrs prior to the assay, target cells were resuspended at 3×10^5 /ml in RPMI/10% FCS in the presence or absence of 3 μ g/ml β_2 microglobulin and 10 μ g/ml total peptide. After preincubation, target cells were centrifuged and pellets were resuspended in 200 μ l (300 μ Ci) sodium (⁵¹Cr) chromate (NEN).

35

Cells were incubated at 37°C for 1 hr with agitation.

Labelled target cells were washed 3 times with RPMI/10% FCS.

c. Setting-Up the Assays: Target cell concentration was adjusted to 10^5 /ml in RPMI/10% FCS and 100 μ l aliquots were added to each well containing responders. K562 cells (cold targets, to block NK, and LAK activity) were washed and resuspended in RPMI/10% FCS at 10^7 /ml. Aliquots of 20 μ l were added per well, yielding a 20:1 of cold K562 target:labelled target. For the determination of the spontaneous ^{51}Cr release, 100 μ l/well of RPMI/10% FCS were added to 100 μ l/well of labelled target cells, and 20 μ l/well of K562. For maximum ^{51}Cr release, 100 μ l 1% Triton X-100 (Sigma) in PBS CMF, was added to the 100 μ l/well labelled target cells, and 20 μ l/well K562. Plates were centrifuged for 2 min at 1200 rpm to accelerate cell conjugate formation. Assays were incubated for 5 hr at 37°C, 5% CO_2 . Assays were harvested by centrifuging plates for 5 min at 1200 rpm and collecting 100 μ l/well of supernatant. Standard gamma counting techniques were used to determine percent specific lysis (Micromedic automatic gamma counter, 0.5 min per tube).

Cultured Cell Lines. JY, a HLA A2.1 expressing human EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562, a NK cell sensitive erythroblastoma line was grown in RPMI/10% FCS. K562 was used to reduce background killing by NK and LAK cells in the chromium release assays.

Peptides. The peptides used in these studies were synthesized at Cytel and their sequences are described in Table 24 a. Peptides were routinely diluted in 100% DMSO at 20 mg/ml, aliquoted, and stored at -20°C.

FACS Analysis. Approximately 10^6 cells were used for each antibody that was to be tested. Cells were washed twice with PBS CNU + 0.1% BSA. To each sample, 100 μ l PBS CMF + 0.1% BSA + primary antibody at 2 μ g/ml (BB7.2, ATCC) or (9.12.1, Inserm-CNRS, Marseille, France) or (LB3.1, Children's Hospital Pittsburgh) were added. A negative control was always

included. Cells were incubated on ice for 20 min and washed twice with PBS CMF + 0.1% BSA. Cells were resuspended in 100 μ l anti-mouse IgG FITC conjugate (Sigma), diluted 1:50 in PBS CMF + 0.1% BSA, and incubated 20 min on ice. Cells were washed twice with PBS CMF + 0.1% BSA, and resuspended in PBS for FACScan (Becton Dickinson) analysis. When it was necessary to postpone analysis to the subsequent days, the cells were fixed with PBS/1% paraformaldehyde (Fisher) and analyzed within one week.

Binding Assays Using Intact Cells and Radiolabelled Peptide.

JY cells were treated with citrate-phosphate buffer and neutralizing buffer #1 as described above. JY control cells were left untreated in tissue culture media. After treatment both cell populations were washed twice with serum free RPMI and loaded with 125 I-radiolabelled 941.01 (HBc15-27) peptide (standard chloramine T iodination). To determine binding specificity, 2×10^6 cells were resuspended in 200 μ l neutralizing buffer #2 (described above) containing 125 I-941.01 (10^5 cpms) +/- 100 μ g unlabelled 941.01. Cells were incubated for 4 hrs at 20°C and washed twice with serum free RPMI to remove free peptide. Cells were resuspended in 200 μ l of serum free RPMI. In a microfuge tube the cell suspension was layered over an 800 μ l FCS and pelleted by centrifugation for 5 sec. Supernatants were aspirated and the radioactivity remaining in the pellet was measured (Micromedic automatic gamma counter, 1 min per tube).

Example 15

Class I MHC molecule peptide stripping/loading by mild acid treatment.

Mild acid solutions of pH 3 such as glycine or citrate-phosphate buffers have been used by various groups to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is unique in that only the MHC class I molecules are destabilized (and peptides released), while all other surface antigens remain intact including MHC class II molecules. Most importantly, treatment of cells with

the mild acid solutions of this example do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of endogenous peptides occurs in two minutes at 4°C and the APC is ready to perform its function after the appropriate peptides are loaded. In this example we utilized the technique to make peptide specific APCs for the generation of primary antigen-specific CTL. The resulting APC were efficient in inducing peptide-specific CD8+ CTL.

Measurements by FACS Analysis. PHA-induced T-cell blasts were acid stripped/peptide loaded according to the methods described in Example 15. The resulting cells were stained for FACS analysis using anti-HLA-A2 (BB7.2) and anti-HLA alpha chain-specific (9.12.1) monoclonal antibodies. Controls for this experiment included the same cell population which was not treated at pH 3 (but treated with PBS buffer at pH 7.2), and with cells treated with citrate-phosphate buffer (to strip the MHC) but neutralized in the absence of β_2 microglobulin and peptide. The results presented in Figure 15, indicate that treatment of these cells with the citrate-phosphate (pH3) buffer significantly reduced (10-fold) the reactivity of the cells toward both anti-HLA class I antibodies alone (anti-HLA-A2 and the alpha chain specific), but not towards a monoclonal antibody specific for class II MHC molecules (anti-HLA-DR). Most importantly, neutralization of the acid-stripped cells in the presence of β_2 microglobulin and peptide resulted in preservation of a significant amount of class I MHC antibody-reactive sites, with only a 2.5-fold decrease in fluorescence intensity. Importantly, the acid-treated cells remained viable, as measured by trypan blue exclusion and forward/lateral FACS scatter analysis. Similar results were obtained using EBV-transformed B cell lines, fresh (or frozen) PBMC and other peptides (which bind to either HLA-A2.1 or HLA-A1) (data not shown).

Binding of Radiolabeled Peptides to Empty MHC Molecules. To determine the efficiency of peptide loading using the cold

temperature incubation or acid stripping/peptide loading protocol, JY cells (an HLA-A2.1 EBV-transformed B cell line) were preincubated at 26°C overnight or acid-stripped to remove the endogenous MHC-associated peptides and the loading of exogenous peptide was determined using a ¹²⁵I-radiolabelled HLA-A2.1 binding peptide. The specificity of this reaction was determined by measuring the inhibition of labelled peptide binding using a cold peptide of the same sequence. Results presented in Table 24 b demonstrate that acid-treatment of the cells increased significantly (approximately 10-fold) the amount of labelled peptide binding to the JY cells. Furthermore, the binding of labelled peptide was completely blocked by the addition of the cold peptide, demonstrating specific binding (data not shown).

In Vitro Induction of Primary Antigen-Specific CTL Using Acid Stripped/Peptide Loaded APCs. Additional critical parameters for the induction of primary CTL using both the cold temperature incubation and acid strip protocol are: 1) enrichment of CD8+ T-cells in the responder cell population (or depletion of CD4+ T-cells), 2) addition of rIL-7 to the CTL induction cultures from day 0, and 3) restimulation of the cultures with antigen on day 12-14 using autologous adherent cells pulsed with peptide. Results presented in Figs. 16 and 17 show experiments performed using PBMC and PHA-induced T-cell blasts as APC. Figure 18 shows experiments using PHA-induced T-cell blasts as APC while Figure 19 shows the use of PBMC as APC.

Example 16

Screening peptides to identify CTL epitopes.

In order to identify CTL epitopes, CTL was stimulated by SAC-I activated PBMCs as APC. Cold temperature expression of the MHC in which class I β -2-microglobulin complex is unstable was utilized in addition to acid stripping to generate PBMC APC.

Complete Culture Medium. The tissue culture medium used in this study consisted of RPMI 1640 with Hepes and L-glutamine (Gibco) supplemented with 2 mM L-glutamine (Irvine Scientific), 0.5mM sodium pyruvate (Gibco), 100 U/100 ug/ml penicillin/streptomycin (Irvine), and 5% heat-inactivated Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture media used in the growth of EBV-transformed lines contained 10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine) instead of human serum.

Cytokines. Recombinant human Interleukin-2 (rIL-2) and Interleukin-4 (rIL-4) were obtained from Sandoz and used at a final concentration of 10 U/ml and 10 ng/ml, respectively. Human interferon- γ (IFN- γ) and recombinant human Interleukin-7 (rIL-7) were obtained from Genzyme and used at 20 U/ml and 10 ng/ml, respectively.

Peptides. Peptides were synthesized at Cytel and are described in Table 24 a. Peptides were routinely diluted in 100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until use.

Cell Lines. JY, Steinlin, EHM, BVR, and KT3 are homozygous human EBV-transformed B cell lines expressing HLA A_{2.1}, A₁, A₃, A₁₁, and A₂₄, respectively. They are grown in RPMI/10% FCS. K562, an NK cell sensitive, erythoblastoma line grown in RPMI/10% FCS, was used for reduction of background killing in CTL assays. Melanoma cell lines either expressing the MAGE antigen, mel 397 and mel 938, or not expressing the MAGE antigen; mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Whole blood was collected into heparin containing syringes and spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15 minutes. The plasma layer was then removed and 10 ml of buffy coat was collected with a pipette using a circular motion. The buffy coat was mixed well and diluted with an equal volume of RPMI. The buffy coat (30 ml) was then layered on 20 ml of

Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg) for 20 minutes, 25°C, with the brake off. The interface between the Ficoll and the plasma containing the PBMCs was recovered with a transfer pipet (two interfaces per 50 ml tube) and washed three times with 50 ml of RPMI (1700, 1500, and 1300 RPM for 10 minutes). Cells were resuspended in 10-20 ml of culture medium, counted, and adjusted to the appropriate concentration.

- 10 **Freezing PBMCs.** 30 million cells/tube (90% FCS/10% DMSO; Sigma) were inserted into a Nalgene Cryo 1°C Freezing Container containing isopropanol (Fisher) and placed at -70°C from 4 hrs (minimum) to overnight (maximum). The isopropanol was changed every five times. Tubes were transferred to liquid nitrogen for long term storage. To thaw, PBMCs were continuously shaken in a 37°C water bath until the last crystal was almost thawed (tubes were not allowed to sit in the water bath or at room temperature for any period of time). Cells were diluted into serum-free RPMI containing 30 µg/ml DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs

- a. Preparation of APCs: PBMCs were purified using the standard Ficoll-Paque protocol and resuspended at 1×10^6 /ml in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells expressing Protein A; Calbiochem), 20 µg/ml Immunobeads (Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4. Two ml of cells per well were plated in a 24-well plate (Falcon, Becton Dickinson) and cultured at 37°C. After 3 days, the medium was removed and the cells were washed three times followed by addition of RPMI/10% HS. The cells were used after culturing for an additional 2 days in RPMI/10% HS.

- b. Expression of empty Class I molecules on the surface of APCs and peptide loading of APCs.

1. Cold temperature incubation:

- a. Expression of empty MHC in APCs: The APCs

were adjusted to a concentration of 2×10^6 /ml in complete culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN- γ , and 3 μ g/ml β_2 -microglobulin (β_2 m; Scripps Lab). The cells were then incubated overnight at 26°C in the presence of 5% CO₂. It should be noted that these cells only express a fraction of Class I molecules in the empty state (~10%).

b. Peptide loading of APC stimulator cells:

Empty Class I expressing APCs were washed 1-2 times with serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1×10^7 in serum-free RPMI containing 50 μ g/ml total of the peptide pool (i.e., 16.7 μ g/ml of each peptide in a pool of three; 25 μ g/ml of each peptide in a pool of two; 50 μ g/ml of individual peptide), 30 μ g/ml DNase, and 3 μ g/ml β_2 m. Following a 4 hour incubation at 20°C, the cells were irradiated at 6100 rads (5×10^6 / ml; 25 million cells/tube), washed and adjusted to the appropriate concentration for addition to the induction culture (see below).

2. Acid stripping: This was used as an alternative method for generating empty MHC on the surface of the APCs. The SAC-I activated PBMCs were washed once in cold 0.9% sodium chloride (J.T. Baker) containing 1% BSA. The cells were resuspended at 10^7 /ml in cold citrate-phosphate buffer (0.13M L-ascorbic acid [J.T. Baker], 0.06M sodium phosphate monobasic [Sigma], pH3) containing 1% BSA and 3 μ g/ml β_2 m and incubated on ice. After 2 minutes, 5 volumes of cold 0.15M sodium phosphate monobasic buffer, pH7.5, containing 1% BSA, 3 μ g/ml β_2 m, and 10 μ g/ml peptide [neutralizing buffer #1] was added and the cells centrifuged at 1500 RPM for 5 minutes at 4°C. The cells were resuspended in 1 ml of cold PBS containing 1% BSA, 30 μ g/ml DNase, 3 μ g/ml β_2 m, and 50 μ g/ml peptide [neutralizing buffer #2] and incubated for 4 hours at 20°C. As above, subsequent to the four hour incubation at 20°C, the cells were irradiated at 6100 rads (5×10^6 / ml; 25 million cells/tube), washed, then adjusted to the appropriate concentration for addition to the induction culture (see below).

c. Preparation of the CD4+ depleted PBMC responder cell population (depletion of lymphocyte sub-populations using AIS flasks). AIS MicroCollector T-150 flasks (specific for the depletion of CD4+ T cells; Menlo Park, CA) were primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30 seconds so that all surfaces were moistened, and then incubating with the binding surface down at room temperature for 1 hour. Following this incubation, flasks were shaken vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2 additional times with PBS and then incubated with 25 ml of culture medium for 15 minutes. PBMCs were thawed in serum-free RPMI (+ L-glutamine + Hepes) containing 30 μ g/ml DNase, washed once, and incubated for 15 minutes in culture medium. Following aspiration of culture medium from the flasks, up to 180 million PBMCs were added in 25 ml of culture medium containing 30 μ g/ml DNase. After 1 hour at room temperature, the flasks were rocked gently for 10 seconds to resuspend the nonadherent cells. The nonadherent cell suspension containing the CD8+ T cells was collected and the flasks were washed 2 times with PBS. The CD4+ T cell depleted PBMCs were centrifuged and counted for addition to the induction culture. The CD4+ and CD8+ phenotype of the CD4+ depleted cell population was determined by FACS analysis (see below). In general, this technique resulted in a two-fold enrichment for CD8+ T cells with an average of approximately 40-50% CD8+ T cells and 15-20% remaining CD4+ T cells following depletion of CD4+ T cells. Depletion of CD4+ T cells can also be accomplished by antibody and complement or antibody coated magnetic beads (Dynabeads). Depletion of CD4+ T cells served the purpose of enriching CTLp and removing cells which would compete for cell nutrients and may interfere with CTLp expansion.

d. Induction of primary CTL. During the 4 hour peptide loading of the stimulator APCs, CD4+ depleted PBMC to be used as the responder population were prepared utilizing AIS flasks for selection of CD8+ T cells through the depletion of CD4+ T cells (above). The responder cells were plated at 3×10^6 /ml in a 1 ml volume (24 well plate) and placed at 37°C until the

peptide loaded stimulator APCs were prepared. The irradiated, peptide loaded APCs were washed 1 time in serum-free RPMI (+ L-glutamine and Hepes), adjusted to 1×10^6 /ml in complete medium, and plated into a 24 well plate at 1 ml/plate: For PBMC, 1×10^6 stimulator cells (1 ml volume) were plated into the wells containing the responder cells; For SAC-I activated PBMC and PHA blasts, 1 ml of 3×10^5 /ml stimulator cells were plated in each well. A final concentration of 10 μ g/ml of additional peptide was added in addition to 10 ng/ml final concentration of rIL-7 (2 ml total volume). On day 7 an additional 10 μ g/ml rIL-7 was added to the culture and 10 U/ml rIL-2 was added every 3 days thereafter. On day 12, the cultures were restimulated with peptide pulsed adherent cells and tested for cytolytic activity 7 days later (below).

Protocol for Restimulation of Primary CTL Using Adherent APC. PBMCs were thawed into serum-free RPMI (+ L-glutamine and Hepes) containing 30 μ g/ml DNase, washed 2 times, and adjusted to 5×10^6 /ml in culture medium containing DNase. PBMCs (25 million cells/tube in 5 ml) were irradiated at 6100R. After 1 wash, the PBMCs were resuspended in culture medium and adjusted to 4×10^6 /ml. 1 ml of irradiated PBMCs was added per well of a 24-well plate. The PBMC were incubated for 2 hours at 37°C, washed 3 times to remove non-adherent cells, and cultured in medium containing 20 μ g/ml total peptide and 3 μ g/ml β_2 microglobulin added in a 0.5 ml volume and again incubated for 2 hours at 37°C. The peptide was aspirated and 1.5×10^6 responder cells resuspended in culture medium were added in a 1 ml volume. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added.

FACS Analysis. One million cells/tube were centrifuged, resuspended in 100 μ l/tube PBS/0.1% BSA/0.02% sodium azide (Sigma) plus 10 μ l/tube directly conjugated antibody (Becton Dickinson), and incubated on ice 15-20 minutes. Cells were then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and resuspended in PBS to analyze on FACScan (Becton Dickinson). When it was not possible to analyze samples within 1-2 days,

cells were fixed with PBS containing 1% paraformaldehyde (Fisher) and analyzed within one week.

Cytotoxicity Assay

5 a. Target cell preparation. Approximately 16-20 hours prior to the CTL assay, target cells (Class I matched EBV-transformed lines) were washed once and resuspended in a 10 ml volume at 3×10^5 /ml in RPMI/5% FCS in the presence or absence of 10 μ g/ml total peptide.

10 b. Labeling of target cells: Target cells were centrifuged and resuspended in 200 μ l/tube sodium ^{51}Cr chromate (NEN), then incubated at 37°C for 1 hour on a shaker. Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and resuspended in 10 ml (to determine the efficiency of labelling, 50 μ l/target was counted on the Micromedic automatic gamma counter).

15 c. CTL assay. Target cells were adjusted to 2×10^5 /ml and 50 μ l of the cell culture was added to each well of a U-bottomed 96-well plate (Costar Corp.) for a final concentration of 1×10^4 /well. K562 cells were washed once, resuspended at 4×10^6 /ml, and 50 μ l/well was added for a final concentration of 2×10^5 /well (ratio of cold K562 to target was 20:1). Responder cells were washed once, resuspended at 9×10^6 /ml, and three fold serial dilutions were performed for effector to target ratios of 90:1, 30:1, 10:1, and 3:1. Responder cells were added in a volume of 100 μ l in duplicate wells. For spontaneous release, 50 μ l/well of labelled target cells, 50 μ l/well K562, and 100 μ l/well of medium was added. For maximum release, 50 μ l/well target, 50 μ l/well K562, and 100 μ l/well of 0.1% Triton-X100 (Sigma) was added. Plates were centrifuged for 5 minutes at 1200 RPM. Following a 5 hour incubation at 37°C, plates were centrifuged again for 5 minutes at 1200 RPM, and 100 μ l/well of supernatant was collected. Standard gamma counting techniques (Micromedic automatic gamma counter; 0.5 minutes/tube) were used to determine the percent specific lysis according to the formula: % specific lysis = $\frac{\text{cpm experimental} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100$.

A cytotoxicity assay (CTL assay) was considered positive if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 15% greater than lysis of control targets (i.e., target cells without peptide). A cytotoxicity assay (CTL assay) was considered borderline if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T ratios was 6% greater than lysis of control targets (i.e., target cells without peptide).

d. Results. Of the peptides that bind to the indicated alleles, 9 of the 49 MAGE peptides, 10 of the 45 HIV peptides, 3 of the 25 HCV peptides, and 2 of the 20 HBV peptides tested to date induced primary CTL *in vitro*. Representative graphs illustrating CTL responses to various immunogenic peptides are shown for MAGE (Figure 22), HIV (Figure 23), HCV (Figure 24), and HBV (Figure 2). The CTL induction data are summarized in Table 24 which lists the immunogenic peptides which bind to the appropriate MHC and induce primary CTL *in vitro*. Indicated is the peptide's sequence, corresponding antigen and HLA allele to which it binds. Results shown in Figure 20 illustrate lysis of peptide sensitized targets and endogenous targets following stimulation with SAC-I activated PBMCs loaded with a MAGE 3 peptide, 1044.07 by the cold temperature and incubation technique. Figure 21 shows a comparison of the acid strip loading technique (Panel a) with the cold temperature incubation technique (panel b).

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

TABLE 13

VALIDATION OF CYTEL'S HLA MOTIFS

5

Binding Capacity (IC ₅₀ nM)						
Sequence	Motif	A1	A2.1	A3.2	A11	A24
AADKAAAAY	A1	50	--*	--	--	--
ATAKAAAAY	A1	15	--	329	77	--
ATDKAAAAY	A1	2.8	--	9250	840	ND
0 ALAKAAAV	A2.1	--	125	--	--	--
AMAAAAAAK	A3.2	--	--	48	8.4	--
ATAAAAAAK	A11	--	--	59	40	--
AYAKAAAF	A24	--	--	--	--	115

15 *A dash indicates an IC₅₀ greater than 20,000 nM.

TABLE 14

VALIDATION OF CYTEL'S HLA MOTIFS

20

20	Binding Capacity (IC ₅₀ nM)					
	SEQUENCE	MOTIF	A1	A2.1	A3.2	A11
	AADKAAAAAY	A1	45	--*	--	--
25	ATAKAAAAAY	A1	58	--	1100	1030
	ATDKAAAAAY	A1	4.0	--	10000	4533
	ALAKAAAAV	A2.1	ND	1400	--	--
	AMAAAAAAK	A3.2	ND	--	85	24.0
30	ATAAAAAAK	A11	--	--	216	88

*A dash indicates an IC₅₀ greater than 20,000 nM.

89

TABLE 15

HLA-A3.2

	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
5	952.25	ALAAAAAAK	1	.
	952.26	AMAAAAAAK	1.2	position 2
	952.23	AVAAAAAAK	0.95	
	981.04	ASAAAAAAK	0.89	
10	952.24	AIAAAAAAK	0.57	
	952.27	AAAAAAA K	0.57	
	981.06	ATAAAAAAK	0.49	
	981.08	AFAAAAAAK	0.13	
	981.09	AGAAAAAAK	0.077	
15	981.13	ACAAAAAAK	0.031	
	981.12	ADAAAAAAK	0.014	
	981.11	ANAAAAAAK	0.0010	
	981.05	AKAAAAAAK	<0.0016	
	981.07	AYAAAAAAK	<0.0005	
20	981.10	APAAAAAAK	<0.0006	
	952.35	ALAAAAAAR	0.46	position 9
	981.36	ALAAAAAAY	0.15	
	981.33	ALAAAAAAA	0.0034	
	981.35	ALAAAAAAQ	<0.0006	
25	981.37	ALAAAAAAS	<0.0005	
	981.38	ALAAAAAAT	<0.0005	
	981.34	ALAAAAAAN	<0.0005	
	981.39	ALAAAAAAE	<0.0003	

90

TABLE 16

HLA-A11

5		PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
		952.25	ALAAAAAAK	1	-
		952.26	AMAAAAAAK	2.5	position 2
		952.27	AAAAAAAK	1.1	
		952.24	AIAAAAAAK	0.72	
10		981.06	ATAAAAAK	0.55	
		981.04	ASAAAAAAK	0.46	
		981.09	AGAAAAAAK	0.38	
		952.23	AVAAAAAAK	0.23	
		981.11	ANAAAAAAK	0.23	
15		981.13	ACAAAAAAK	0.019	
		981.08	AFAAAAAAK	0.020	
		981.12	ADAAAAAAK	0.012	
		981.05	AKAAAAAAK	0.0065	
		981.07	AYAAAAAAK	<0.0065	
20		981.10	APAAAAAAK	<0.0051	
		952.35	ALAAAAAAR	0.015	position 9
		981.33	ALAAAAAAA	<0.0059	
		981.34	ALAAAAAAN	<0.0071	
		981.35	ALAAAAAAQ	<0.0051	
25		981.36	ALAAAAAAY	<0.0071	
		981.37	ALAAAAAAS	<0.0051	
		981.38	ALAAAAAAT	<0.0051	
		981.39	ALAAAAAAE	<0.0071	

91

TABLE 17

HLA-A24

5		PEPTIDE	SEQUENCE	AVERAGE RATIO TO 983.01	SUBSTITUTION
		983.01	AYAKAAAF	1	-
		983.08	AFAKAAAF	0.24	position 2
		983.09	APAKAAAF	0.0058	
		983.10	AAAKAAAF	0.0023	
10		983.11	AKAKAAAF	<0.0012	
		983.05	AYAKAAAI	0.20	position 9
		983.04	AYAKAAAL	0.11	
		983.06	AYAKAAAV	0.0023	
		983.02	AYAKAAAA	<0.0012	
15		983.03	AYAKAAAY	<0.0012	
		983.07	AYAKAAAK	<0.0012	

TABLE 18

HLA-A1

5	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
	982.011	ATDKAAAAAY	Motif	-
	982.07	ATAKAAAAAY	1	-
	982.09	ASAKAAAAAY	0.17	position 2
	982.13	AMAKAAAAAY	0.095	no D in pos 3
10	982.08	AAAKAAAAAY	0.0064	
	954.09	ALAKAAAAAY	0.0045	
	954.11	AIKAAAAAY	0.0045	
	954.13	AVAKAAAAAY	0.0020	
	982.10	AKAKAAAAAY	0.0011	
15	982.11	ANAKAAAAAY	<0.0001	
	982.12	ADAKAAAAAY	<0.0001	
	982.14	AGAKAAAAAY	<0.0001	
	982.15	APAKAAAAAY	<0.0001	
	982.16	AYAKAAAAAY	<0.0001	
20	982.17	AHAKAAAAAY	<0.0001	
	982.24	ATAKAAAAA	0.0040	position 9
	982.23	ATAKAAAAF	0.0019	no D in pos 3
	982.28	ATAKAAAAH	0.0010	
	982.32	ATAKAAAAV	0.0005	
25	982.25	ATAKAAAAAN	<0.0001	
	982.26	ATAKAAAAAD	<0.0001	
	982.27	ATAKAAAAW	<0.0001	
	982.30	ATAKAAAAK	<0.0001	
	982.31	ATAKAAAAI	<0.0001	
30	982.29	ATAKAAAAAP	<0.0001	

TABLE 19

HLA-A1

PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
982.01	ATDKAAAAAY	Motif	-
982.07	ATAKAAAAAY	1	-
982.01	AADKAAAAAY	0.14	position 3
954.03	AAEKAAAAAY	0.038	no T in pos 2
982.02	AAAKAAAAAY	0.0055	
982.06	AASKAAAAAY	0.0024	
982.04	AANKAAAAAY	0.0011	
982.03	AAQKAAAAAY	0.0008	
982.05	AAKKAAAAAY	<0.0001	
982.20	AADKAAAAA	0.0016	position 9
982.21	AADKAAAAW	0.0005	no T in pos 2
982.19	AADKAAAAF	<0.0001	
982.22	AADKAAAAK	<0.0001	

TABLE 20 (A)

HPV16 E6 AND E7 PEPTIDES BINDING TO HLA-A1

5	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
	E6	80	ISEYRHYAY	3.500	+
	E6	69	VADKALKFY	0.240	+
	E7	44	QAEPDRAHY	0.029	+
10	E7	37	EIDGPAGQA	0.025	-
	E7	19	TTDLYAYEQ	0.023	+
	E6	144	MSAARSSRT	0.019	+/-
	E7	73	HVDIRTLED	0.014	-
	E6	139	WTGRAMSAA	0.010	-
15	E6	61	YRDGNPYAV	0.008	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

† The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 81±30 nM. Listed in the table are peptides yielding ratio values of ≥0.001. All other peptides yielded ratio values of <0.001.

TABLE 20(B)
HPV16 E6 and E7 Peptides Binding to HLA-A3.2

	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
5	E6	107	LIRAINAQK	3.7000	+
	E6	59	IVYRDGNPY	3.0000	+
	E7	89	IVAPIASQK	2.2000	+
10	E6	33	IILEAVYAK	1.5000	+
	E6	125	HLDKKQRFH	0.4400	+
	E6	143	AMSAARSSR	0.1800	+
	E6	7	AMFQDPQER	0.1000	+
	E6	93	TTLEQQYNK	0.0780	+
15	E6	37	AVYAKQQLL	0.0320	-
	E7	51	HYNIVTFAA	0.0210	-
	E6	145	SAARSSRTR	0.0200	+
	E6	75	KFYISKISEY	0.0100	+
	E6	89	SLYGTTLQ	0.0080	-
20	E7	52	YNIVTFAAK	0.0067	-
	E6	80	ISEYRHYAY	0.0064	+
	E6	42	QQLLRREYV	0.0058	-
	E6	68	AVADKALKF	0.0056	+
	E6	97	QQYNKPLAD	0.0045	-
25	E6	79	KISEYRHYA	0.0044	-
	E6	84	RHYAYSLYG	0.0036	-
	E6	69	VADKALKFY	0.0025	+
	E6	146	AARSSRTRR	0.0020	+
	E7	58	AAKADSTLR	0.0016	+
30	E6	38	VYAKQQLLR	0.0012	-
	E6	67	YAVADKALK	0.0012	+
	E7	60	KADSTLRLA	0.0012	-

* Bold A's indicate residues in which cysteine was replaced by alanine.
 † The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 30±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of ≤0.001.

Table 20(C)
HPV16 E6 and E7 Peptides Binding to HLA-A11.2

	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
5	E6	33	IILEAVYAK	6.7000	+
	E6	93	TTLEQQYNK	1.8000	+
	E7	89	IVAPIASQK	1.3000	+
10	E6	7	AMFQDPQER	0.8400	+/-
	E6	59	IVYRDGNPY	0.4700	- (+)‡
	E6	80	ISEYRHYAY	0.4300	- (+)‡
	E6	37	AVYAKQQLL	0.0450	-
	E6	145	SAARSSRTR	0.0330	+/-
15	E6	107	LIRAINAQK	0.0120	+
	E7	58	AAKADSTLR	0.0110	+/-
	E6	42	QQLLRREVY	0.0084	+/- (+)‡
	E6	143	AMSAARSSR	0.0084	-
	E6	79	KISEYRHYA	0.0076	-
20	E6	67	YAVADKALK	0.0074	+
	E7	52	YNIVTFAAK	0.0060	+
	E6	68	AVADKALKF	0.0037	-
	E6	69	VADKALKFY	0.0030	- (+)‡
	E6	38	VYAKQQLLR	0.0022	+/-
25	E6	140	TGRAMSAAR	0.0012	+/-
	E7	90	VAPIASQKP	0.0012	-
	E7	51	HYNIVTFAA	0.0010	-

* Bold A's indicate residues in which cysteine was replaced by alanine.
† The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 10±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of <0.001.
‡ Brackets indicate score according to adjusted motif.

Table 20(D)
HPV16 E6 and E7 Peptides Binding to HLA-A24

5	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
	E6	87	AYS L YGTT L	0.1200	+
	E6	72	KALKFY S KI	0.1100	- (+) §
	E6	131	R F H N IRGRW	0.1000	+
10	E7	49	RAHYNIVTF	0.0670	- (+) §
	E6	49	VYDFAFRDL	0.0610	+
	E6	82	EYRHYAYS L	0.0460	+
	E6	26	LQTTIHDI I	0.0200	-
	E6	66	PYAVADK A L	0.0055	-
15	E6	1	M H QKRTAMF	0.0049	-
	E6	85	HYAYS L YGT	0.0037	-
	E6	44	LLRRE V YDF	0.0023	+
	E6	38	VYAKQ Q LLR	0.0011	-

- * Bold A's indicate residues in which cysteine was replaced by alanine.
- † The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 22±6 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of <0.001.
- § Brackets indicate score according to adjusted motif.

TABLE 20 (B)
SUMMARY OF EFFICACY OF 2, (3), 9 ANCHOR MOTIFS

Percentage Binders With Motifs	HLA Allele			
	A1	A3.2	AI1.2	A24
	Predicted/Found	Predicted/Found	Predicted/Found	Predicted/Found
High (≥ 0.1)	2 2(100%)	7 7(100%)	6 6(100%)	2 3(67%)
Intermediate (0.1-0.01)	3 6(50%)	3 5(60%)	3 4(75%)	2 4(50%)
Weak (0.01-0.001)	0 1(0%)	6 14(43%)	6 11(55%)	1 5(20%)
Negative (≤ 0.001)	7 231(3%)	16 214(7%)	14 219(6%)	2 228(1%)
Totals	12 240	32 240	26 240	7 240

TABLE 21(A)
A3.2 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
IK	3(15%)	4(20%)	7(35%)	6(30%)	20(100%)
IR	3(15.8%)	4(21.1%)	7(36.8%)	5(26.3%)	19(100%)
LK	13(48.1%)	6(22.2%)	5(18.5%)	3(11.1%)	27(100%)
LR	4(8.5%)	9(19.1%)	20(42.6%)	14(29.8%)	47(100%)
SK	0	0	0	0	0
SR	0	0	0	1(100%)	1(100%)
TK	3(15%)	6(30%)	7(35%)	4(20%)	20(100%)
TR	2(16.7%)	1(6.0%)	2(16.7%)	7(58.3%)	12(100%)
VK	9(28.1%)	13(40.6%)	9(28.1%)	1(3.1%)	32(100%)
VR	2(7.4%)	3(11.1%)	14(51.9%)	8(29.6%)	27(100%)
Total	39(19%)	46(22.4%)	71(34.6%)	49(23.9%)	205(100%)

TABLE 21 (B)
All 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
GK	0	1 (100%)	0	0	1 (100%)
IK	5 (25%)	5 (25%)	7 (35%)	3 (15%)	20 (100%)
LK	6 (22.2%)	10 (37%)	9 (33.3%)	2 (7.4%)	27 (100%)
TK	10 (50%)	4 (20%)	4 (20%)	2 (10%)	20 (100%)
VK	12 (37.5%)	15 (46.9%)	4 (12.5%)	1 (3.1%)	32 (100%)
Total	33 (33%)	35 (35%)	24 (24%)	8 (8%)	100 (100%)

101

TABLE 22 (A)
A3.2 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
AR	0	0	1(100%)	0	1(100%)
FK	0	0	0	0	0
FR	0	0	1(25%)	3(75%)	4(100%)
IK	0	6(27.3%)	10(45.5%)	6(27.3%)	22(100%)
IR	1(7.1%)	1(7.1%)	2(14.2%)	10(71.4%)	14(100%)
LK	16(53.3%)	7(23.3%)	5(16.7%)	2(6.7%)	30(100%)
LR	4(12.5%)	9(28.1%)	11(34.3%)	8(25%)	32(100%)
MX	1(100%)	0	0	0	1(100%)
MR	1(100%)	0	0	0	1(100%)
TK	2(11.8%)	5(29.4%)	8(47.1%)	2(11.8%)	17(100%)
TR	1(4.8%)	1(4.8%)	9(42.9%)	10(47.6%)	21(100%)
VK	7(35%)	4(20%)	5(25%)	4(20%)	20(100%)
VR	0	6(21.4%)	15(53.6%)	7(25%)	28(100%)
Total	34(17.5%)	40(20.6%)	68(35.1%)	52(26.8%)	194(100%)

TABLE 22(B)
All 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1 (33.3%)	1 (33.3%)	1 (33.3%)	0	3 (100%)
CK	0	0	1 (100%)	0	1 (100%)
GK	0	0	0	0	0
IK	4 (18.2%)	5 (22.7%)	12 (54.5%)	1 (4.5%)	22 (100%)
LK	9 (30%)	12 (40%)	8 (26.7%)	1 (3.3%)	30 (100%)
MK	1 (100%)	0	0	0	1 (100%)
TK	6 (35.3%)	5 (29.4%)	5 (29.4%)	1 (5.9%)	17 (100%)
VK	7 (35%)	8 (40%)	4 (20%)	1 (5%)	20 (100%)
Total	28 (29.8%)	31 (33%)	31 (33%)	4 (4.3%)	94 (100%)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0000	HLDMLRHLY	9	c-ERB2			42	1	9.1	0.007	0.0002	
1.0046	LLDIDETDY	9	c-ERB2			849	1	7.4	0.0003	0	
1.0005	GTQFEDNY	9	c-ERB2			104	1	0.18	0	0.028	
1.0063	LTCSPQPEY	9	c-ERB2			1131	1	0.13	0	0.0061	
1.0017	ETLEETCY	9	c-ERB2			401	1	0.043			
1.0749	FTHQSDVWSY	10	c-ERB2			899	1	2.7	0.0003	0.0005	
1.0747	RLDIDETDY	10	c-ERB2			848	1	1.3	0.0017	0	
1.0715	TLLEETCYLY	10	c-ERB2			402	1	1.1	0	0	
1.0737	YVMAGVGSFY	10	c-ERB2			772	1	1.1	0.010	0.012	0
1.0764	GTFIAENPEY	10	c-ERB2			1239	1	0.063			
1.0708	LQRNPLQCY	10	c-ERB2			154	1	0.030			
1.0603	VVQGNLELY	10	c-ERB2			55	1	0.018			
1.0756	MCDLVDAEY	10	c-ERB2			1014	1	0.012			
1.1028	KIRKYTMRR	9	c-ERB2			681	3.11		0.76	0.0018	
1.1027	VVFGILQK	9	c-ERB2			649	3.11		0.11	0.72	
1.0044	LVKSPNHVK	9	c-ERB2			852	3.11		0.48	0.070	
1.0035	VLRENTSPK	9	c-ERB2			754	3.11		0.40	0.013	
1.0029	ILIKRQOK	9	c-ERB2			673	3.11		0.38	0.0097	
1.0011	ILWKDIFHK	9	c-ERB2			187	3.11		0.28	0.31	
1.1033	KITDFGLAR	9	c-ERB2			840	3.11		0.17	0.24	
1.0849	GVVFGILK	9	c-ERB2			648	3.11		0.0047	0.089	
1.0299	QVCTGTDMK	9	c-ERB2			24	3.11		0.0007	0.052	
1.1081	LLDHVREIR	9	c-ERB2			806	3.11		0.007	<0.0006	
1.1026	CVNCSQFLR	9	c-ERB2			528	3.11		0.0015	0.001	
1.1023	TVCACCCAR	9	c-ERB2			218	3.11		0.0004	0.003	
1.0031	ILKETELK	9	c-ERB2			714	3.11		0.019	0.0023	
1.1024	VTAEDQTOR	9	c-ERB2			322	3.11		<0.0002	0.014	
1.0026	DLSPMPWK	9	c-ERB2			607	3.11		0.0005	0.010	
1.0707	TILWKDIFHK	10	c-ERB2			166	3.11		0.043	3.6	
1.0712	GTQCEKCSK	10	c-ERB2			327	3.11		0.0071	0.61	
1.0736	KVLRENTSPK	10	c-ERB2			753	3.11		0.38	0.22	
1.0702	QUSLTEILK	10	c-ERB2			143	3.11		0.20	0.013	
1.1142	RLVHRLAAR	10	c-ERB2			840	3.11		0.18	0	
1.0741	LLNWCMQAK	10	c-ERB2			822	3.11		0.14	0.24	
1.0752	TIDVYMBIVK	10	c-ERB2			948	3.11		0.013	0.12	
1.0731	RLKETELK	10	c-ERB2			713	3.11		0.007	0.11	
1.0745	VLKSPNHVK	10	c-ERB2			851	3.11		0.002	0.0092	
1.1131	SVFQNLQVIR	10	c-ERB2			423	3.11		0.017	0.075	
1.1133	HTVPWDQLFR	10	c-ERB2			478	3.11		0.0005	0.072	
1.1127	ILKCGVLQK	10	c-ERB2			148	3.11		0.040	0.0005	
1.1143	LVSEFSMAR	10	c-ERB2			972	3.11		0.0072	0.003	
1.1136	GVVFGILK	10	c-ERB2			648	3.11		0.018	0.003	
1.0726	CVARCPGK	10	c-ERB2			596	3.11		0.022	0.0042	
1.1137	VVFGILQK	10	c-ERB2			649	3.11		0.0030	0.016	
1.0728	GLIKRQOK	10	c-ERB2			672	3.11		0.015	0.0014	
1.1129	RTVCACCCAR	10	c-ERB2			217	3.11		0.0048	0.013	
1.1134	CLACHQLCAR	10	c-ERB2			508	3.11		0.011	0	
1.1139	KIPVAIKVLR	10	c-ERB2			747	3.11		0.0009	0.0099	

Table 23(a)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
1.0291	VGEADYFEY	9	EBNA1			409	1	0.016			
1.0295	PUREIVCY	9	EBNA1			553	1	0.010			
1.0681	PVGEADYFEY	10	EBNA1			408	1	0.015			
1.0683	CTWVAGVPVY	10	EBNA1			501	1	0.014			
1.0293	GVPVYQCSK	9	EBNA1			506	3.11		0.30	0.61	
1.1016	KTSLYNLR	9	EBNA1			514	3.11		0.31	0.12	
1.0297	AIKDLVMTK	9	EBNA1			578	3.11		0.048	0.034	
1.0687	QTHIFAELK	10	EBNA1			567	3.11		0.010	0.21	
1.1124	GTALAIPQCR	10	EBNA1			523	3.11		0.0028	0.056	

Table 23(b)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A22	A11	A24
S.0005	CTELKLSQY	9	FLU	A	NP	44	1	3.6			
S.0006	STLELRKY	9	FLU	A	NP	377	1	0.020			
S.0044	ILRGSVAHK	9	FLU	A	NP	265	3		1.5	0.0037	
S.0051	RMGNILKCK	9	FLU	A	NP	221	3		0.27	0.062	
S.0046	LMQGSTLPR	9	FLU	A	NP	166	3		0.031	0.10	
S.0048	MIDGGRFY	9	FLU	A	NP	32	3		0.059	0.0010	
S.0049	MVLSAFDER	9	FLU	A	NP	66	3		0.0016	0.041	
S.0054	YIQMCTELK	9	FLU	A	NP	40	3		0.0031	0.030	
S.0042	GNDRNFWR	9	FLU	A	NP	200	3		0.0028	0.024	
S.0104	SLMQGSTLPR	10	FLU	A	NP	165	3		0.12	0.84	
S.0095	KMIDGGRFY	10	FLU	A	NP	31	3		0.50	0.0079	
S.0096	LILRGSVAHK	10	FLU	A	NP	264	3		0.34	0.037	
S.0102	RSGAAGAAVK	10	FLU	A	NP	175	3		0.019	0.0046	
S.0105	SSTLELRKY	10	FLU	A	NP	376	3		0.0018	0.016	
S.0103	RSRYWAIRTR	10	FLU	A	NP	382	3		0.012	0	
S.0101	RMVLSAFDER	10	FLU	A	NP	65	3		0.0014	0.010	
S.0061	FYQMCTEL	9	FLU	A	NP	39	24				2.9
S.0060	AYERMCNIL	9	FLU	A	NP	218	24				0.031
S.0112	RFYQMCTEL	10	FLU	A	NP	38	24				0.15

Table 23(c)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A1.2	A11	A24
1.0155	LLDTASALY	9	HBV	adr	CORE	420	1	25	0.0007	0	
1.0186	SLDVSAAFY	9	HBV	adr	POL	1001	1	17.2	0.0007	0.0006	
2.0125	PTTGRTSLY	9	HBV	ALL		1382	1	1.3	0.0008	0	
2.0126	MSITDLEAY	9	HBV	adr		1521	1	0.85	<0.0008	0	
1.0208	PTTGRTSLY	9	HBV	adr	POL	1382	1	0.77	0	0	
1.0387	LTKQYLNL	9	HBV	adw	POL	1280	1	0.50	0.0003	0.0075	
2.0122	LTKQYLNL	9	HBV	adw		1280	1	0.095			
1.0166	KVCNPTGLY	9	HBV	adr	POL	629	1	0.068			
2.0127	MSITDLEAY	9	HBV	adw		1550	1	0.067			
2.0120	PSQPSRGNY	9	HBV	ayw		984	1	0.057			
2.0112	PSWAFAYY	9	HBV	adw		316	1	0.054			
2.0119	QSAVRKEAY	9	HBV	adw		881	1	0.025			
1.0174	PLDKGDPY	9	HBV	adr	POL	698	1	0.019			
1.0278	SLMLLYKTY	9	HBV	adw	POL	1092	1	0.017			
2.0115	ASRDLVVS	9	HBV	ayw		499	1	0.013			
2.0124	PSKRLGLY	9	HBV	adr/adw		1364	1	0.011			
2.0121	SSTSRNIN	9	HBV	adr		1036	1	0.0077			
1.0519	DLDTASALY	10	HBV	adr	CORE	419	1	11.1	0	0	
1.0513	LDPRVRGLY	10	HBV	adr	ENV	120	1	6.3	0.17	0	
2.0209	LSLVSAAFY	10	HBV	ALL		1000	1	4.2	<0.0009	0.0007	
1.0911	FLQQYUHL	10	HBV	adr	POL	1250	1	1.1	0.014	0.0048	0.0017
2.0216	QTFGRKLHL	10	HBV	ayw	POL	1087	1	1.1	0.0056	0.012	
2.0244	KTYGRKLHL	10	HBV	adw		1088	1	0.69	0.59	0.22	0
1.0791	KTYGRKLHL	10	HBV	adw	POL	1098	1	0.57	0.53	0.35	0.0001
2.0242	QTFGRKLHL	10	HBV	ayw		1087	1	0.37	0.0037	0.011	
1.0556	KTFGRKLHL	10	HBV	adr	POL	1069	1	0.34	0.094	0.090	0
2.0241	KTFGRKLHL	10	HBV	adr		1069	1	0.30	0.15	0.095	0
1.0766	LQDPVRALY	10	HBV	adw	ENV	120	1	0.21	0.014	0	
1.0806	TTPAQGTSY	10	HBV	adw	ENV	288	1	0.20	0	0	
2.0240	LSSTSRNIN	10	HBV	adr		1035	1	0.20	<0.0009	0	
1.0541	PLDKGDPY	10	HBV	adr	POL	698	1	0.16	0	0	
2.0238	HSASPGCSY	10	HBV	ayw		767	1	0.15	0.019	0.017	0
1.0795	FLTKQYLNL	10	HBV	adw	POL	1279	1	0.12	0	0	
2.0237	RSASPGCSY	10	HBV	adr/adw		738	1	0.11	0.003	0.020	0
1.0774	WLWGMDDPY	10	HBV	adw	CORE	416	1	0.081			
2.0233	TTPAQGTSY	10	HBV	ayw		288	1	0.066			
1.0542	HTLWKAGLY	10	HBV	adr	POL	723	1	0.030			
2.0231	TSQPHICFY	10	HBV	adr		226	1	0.018			
2.0246	KSVQHLSLY	10	HBV	adw		1161	1	0.016			
1.0910	NLYVSLLLY	10	HBV	adr	POL	1059	1	0.015			
1.0901	WMWVWVWPSL	10	HBV	adr	ENV	359	2.1	0.0009	0.019	0	0.0005
2.0089	LLYQTGRK	9	HBV	ayw	POL	1084	3		1.8	0.44	
2.0116	IMFARFYK	9	HBV	ayw		713	3		0.99	1.5	
2.0082	QLHQSPVRK	9	HBV	ayw	POL	867	3		0.14	0.025	
5.0056	SAKSVVRK	9	HBV		POL	531	3		<0.0003	0.067	
2.0077	HLHQDIKK	9	HBV	ayw	POL	686	3		0.061	0.0075	
2.0219	SLPQEHQK	10	HBV	ayw	POL	1197	3		0.36	4.2	
2.0234	SMFPGCCCTK	10	HBV	adr/adw		295	3		0.43	1.9	
2.0235	SMYFPGCCCTK	10	HBV	ayw		295	3		1.1	1.79	
5.0107	QAFTFSPYTK	10	HBV		POL	645	3		0.15	1.3	
2.0214	LLYQTGRK	10	HBV	ayw	POL	1083	3		0.89	0.021	
2.0245	YMDQVVLCAK	10	HBV	ALL		1123	3		0.16	0.0076	
5.0108	TSKSVVRK	10	HBV		POL	530	3		0.0005	0.013	
2.0094	PTYKARLCK	9	HBV	ayw	POL	1263	11		0.000	0.085	
2.0088	PTDLEAYFK	9	HBV	adw	"C"	1552	11		0.0002	0.016	
2.0061	KYTSFFWLL	9	HBV	ALL		1330	24				3.6
2.0059	LYAAVTNFL	9	HBV	adw		1169	24				3.2
2.0046	FYPNLTKYL	9	HBV	adr		689	24				2.1
2.0045	LYSSTVPF	9	HBV	adw/ayw		645	24				1.9
2.0048	FYPKVTIKYL	9	HBV	ayw		718	24				1.7
2.0049	FYPNVTIKYL	9	HBV	adw		718	24				1.6
2.0039	LYSLSPL	9	HBV	ayw		368	24				0.50
2.0044	LYSSTVPVL	9	HBV	adr		636	24				0.37
2.0038	LYNLSPL	9	HBV	adr		368	24				0.34
2.0051	NYRVSWPKF	9	HBV	ayw		991	24				0.18
2.0050	HYPTRIHYL	9	HBV	adw/ayw		743	24				0.15
2.0047	HYFKTRIHYL	9	HBV	adr		714	24				0.057
2.0060	GYPALMPLY	9	HBV	ALL		1224	24				0.049
5.0062	AYRPNAP	9	HBV		NUC:NUCLUS	131	24				0.026
2.0054	LYQTGRKL	9	HBV	ayw		1085	24				0.014
2.0043	SYQHFRLL	9	HBV	ayw		607	24				0.011
2.0181	LYSHPIILCF	10	HBV	ALL		1077	24				1.1
2.0182	LYAAVTNFL	10	HBV	adw		1169	24				0.32
2.0188	LYRPLSLPF	10	HBV	adr		1371	24				0.25

Table 23(d)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0174	SYQHPRKLL	10	HBV	ayw		607	24				0.16
2.0173	SYQHPRKLL	10	HBV	adr/adw		578	24				0.066
2.0176	YYPEHLVNH	10	HBV	ayw		735	24				0.040
2.0172	AYEPFNAPIL	10	HBV	ALL		521	24				0.022
2.0171	GYRWACLERF	10	HBV	ALL		234	24				0.011
3.0115	NRLSLGHL	10	HBV		POL	572	24				0.0099
1.0377	YVSLMLLYK	9	HBV	adw	POL	1080	3.11		0.31	7.4	
1.0189	LLYKTGRK	9	HBV	adr	POL	1066	3.11		5.0	0.30	
1.0379	LLYKTGRK	9	HBV	adw	POL	1085	3.11		2.5	0.40	
1.0370	VTKYLPDK	9	HBV	adw	POL	722	3.11		0.014	1.3	
1.0176	RHYLHLWK	9	HBV	adr	POL	719	3.11		1.2	0.010	
1.0367	STVPSRNP	9	HBV	adw	POL	648	3.11		0.021	0.33	
1.0215	TTDLAYPK	9	HBV	adr	"X"	1523	3.11		0.0006	0.92	
1.0848	YVSLMLLYK	9	HBV	adr	POL	1061	3.11		0.39	0.92	
1.0380	PTYKAPLTK	9	HBV	adw	POL	1274	3.11		0.17	0.71	
1.0987	HLYPVARQR	9	HBV	adr	POL	1257	3.11		0.54	0.0020	
1.0358	STNRQLGRK	9	HBV	adw	ENV	85	3.11		0.51	0.34	
1.0991	ALRFTSARR	9	HBV	adr	"X"	1488	3.11		0.44	<0.0005	
1.0197	PVNRFDWK	9	HBV	adr	POL	1197	3.11		0.080	0.41	
1.0369	TVNENRLLK	9	HBV	adw	POL	703	3.11		0.016	0.40	
1.1041	VVNHVQTR	9	HBV	adw	POL	740	3.11		0.030	0.33	
1.0152	STISGCK	9	HBV	adr	ENV	277	3.11		0.011	0.29	
1.0213	QVPLQLHK	9	HBV	adr	"X"	1505	3.11		0.10	0.28	
1.0172	LTKYLPDK	9	HBV	adr	POL	683	3.11		0.0009	0.23	
1.0374	CLHQSAVRK	9	HBV	adw	POL	878	3.11		0.22	0.017	
1.0980	VYDPSQSR	9	HBV	adr	POL	943	3.11		0.011	0.20	
1.0382	PLYACQAK	9	HBV	adw	POL	1259	3.11		0.18	0.094	
2.0074	YVNTDGLK	9	HBV	ayw	CORE	507	3.11		0.16	0.048	
1.0199	PLYACQSK	9	HBV	adr	POL	1230	3.11		0.11	0.018	
1.0972	RLADEGLNR	9	HBV	adr	POL	601	3.11		0.10	0.025	
1.0976	AVNHVYKTR	9	HBV	adr	POL	711	3.11		0.0071	0.098	
1.0975	RLKLMFAR	9	HBV	adr	POL	680	3.11		0.095	0.0002	
1.0977	ILYKKEITR	9	HBV	adr	POL	730	3.11		0.095	<0.0005	
1.0993	KVPVLCCGR	9	HBV	adr	"X"	1548	3.11		0.042	0.082	
1.0145	NVSPWTHK	9	HBV	adr	POL	621	3.11		0.072	0.076	
1.0982	LLYKTGRK	9	HBV	adr	POL	1065	3.11		0.072	0.0045	
1.0978	RLVQVTSR	9	HBV	adr	POL	757	3.11		0.068	0.0002	
1.0219	PVLCCGRHK	9	HBV	adr	"X"	1550	3.11		0.065	0.019	
1.1042	RLVQVTSR	9	HBV	adw	POL	786	3.11		0.064	0.0002	
1.1043	MLLYKTYGR	9	HBV	adw	POL	1094	3.11		0.061	0.0002	
1.0170	TVNENRLLK	9	HBV	adr	POL	674	3.11		0.048	0.037	
1.1045	NLYPVARQR	9	HBV	adw	POL	1286	3.11		0.042	0.0011	
1.1046	LPYKPTGR	9	HBV	adw	POL	1407	3.11		0.021	0	
1.0845	LVSPGVWR	9	HBV	adr	CORE	508	3.11		0.0003	0.020	
1.0981	LVGSCLPK	9	HBV	adr	POL	1022	3.11		0.0008	0.015	
1.0967	HISCLTGR	9	HBV	adr	CORE	494	3.11		0.013	0.011	
1.1047	SVPSRLPDR	9	HBV	adw	POL	1424	3.11		0.0007	0.010	
1.0989	SVPSRLPDR	9	HBV	adr	POL	1395	3.11		0.0004	0.010	
1.0544	TLQEHIVLK	10	HBV	adr	POL	1179	3.11		0.092	5.6	
2.0205	TVPVNFRWK	10	HBV	ayw	POL	649	3.11		0.0067	4.2	
1.0543	TLWKAGLYK	10	HBV	adr	POL	724	3.11		3.5	1.0	
1.0807	SMYPSOCTK	10	HBV	ayw	ENV	295	3.11		1.5	1.4	
1.1153	RLPYRPTGR	10	HBV	adw	POL	1406	3.11		2.8	0.030	
1.0584	STDLAYPK	10	HBV	adr	"X"	1522	3.11		0.0046	2.7	
1.0554	LLYKTGRK	10	HBV	adr	POL	1065	3.11		2.5	0.012	
1.0799	TVNAHRNLPK	10	HBV	adw	"X"	1529	3.11		0.82	0.45	
1.0546	EAYFKDCLPK	10	HBV	adr	"X"	1527	3.11		0.037	0.74	
1.1081	LVVDFQSR	10	HBV	adr	POL	942	3.11		0.0009	0.43	
1.0789	MLLYKTYGRK	10	HBV	adw	POL	1094	3.11		0.41	0.020	
1.0544	TAYSHLSTK	10	HBV	adr	POL	858	3.11		0.26	0.092	
1.0542	SLGHLNPNK	10	HBV	adr	POL	1150	3.11		0.20	0.078	
1.1152	RLGLYRLLR	10	HBV	adw	POL	1397	3.11		0.19	0.0049	
1.0547	VTGCVFLVDK	10	HBV	adr	POL	943	3.11		0.035	0.17	
1.1150	RIRTPTPAR	10	HBV	adw	POL	942	3.11		0.17	0.0002	
1.0541	TVNGHQVLPK	10	HBV	adr	"X"	1500	3.11		0.073	0.092	
1.1091	SLPQPTTGR	10	HBV	adr	POL	1377	3.11		0.077	0.043	
1.1072	TLPETTVRR	10	HBV	adr	CORE	532	3.11		<0.0003	0.075	
1.1089	GTDSVVLNR	10	HBV	adr	POL	1320	3.11		0.025	0.072	
1.1071	SLPETTVRR	10	HBV	adr	CORE	531	3.11		0.0005	0.068	
2.0210	KVTKYLPDK	10	HBV	ayw	POL	721	3.11		0.027	0.053	
1.1148	STRHGDKSFR	10	HBV	adw	POL	792	3.11		0.0057	0.038	
1.0935	VLSCWWLQFR	10	HBV	adw	POL	923	3.11		0.029	0.0087	
1.0781	NVTKYLPDK	10	HBV	adw	POL	721	3.11		<0.0004	0.023	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A12	A11	A24
1.1092	RVCQOLDPAR	10	HBV	adr	X	1422	3.11		0.0019	0.023	
1.0793	SLGIHLNPOK	10	HBV	adw	POL	1179	3.11		0.017	0.014	
1.0909	YLVSRCVWIR	10	HBV	adr	CORE	508	3.11		0.015	0.0027	
2.0207	FVGPLTVNEK	10	HBV	ayw	POL	698	3.11		0.0057	0.015	
1.0535	YVGPLTVNEK	10	HBV	adr	POL	669	3.11		0.0069	0.014	
1.1075	RLADEGLNRK	10	HBV	adr	POL	601	3.11		0.013	0.0004	
1.1086	IVLKLKQCFK	10	HBV	adr	POL	1185	3.11		0.013	0.0024	
1.0773	PIPSWAFK	10	HBV	adw	ENV	314	3.11		<0.0003	0.010	
1.0778	LTVNENRRLK	10	HBV	adw	POL	702	3.11		0.0025	0.0095	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0118	CTCGSSDLY	9	HCV		LORF	1123	1	3.0	0	0.010	
1.0112	NIVDVQYLY	9	HCV		NS1/ENV2	697	1	0.60	0	0.010	
2.0034	VQDCNCSY	9	HCV			302	1	0.54	0.0005	0.0003	
2.0035	LTPRCMV DY	9	HCV			605	1	0.078			
1.0145	RVCBQMALY	9	HCV		LORF	2588	1	0.053			
1.0140	DVVCSSMSY	9	HCV		LORF	2416	1	0.039			
2.0036	PTTFQIRMY	9	HCV			626	1	0.012			
1.0509	CLSAFSLHSY	10	HCV		LORF	2888	1	0.41	0.013	0.0034	0.0002
1.0499	TLHGPTPLY	10	HCV		LORF	1617	1	0.30	0.11	0.0024	
2.0037	EYVLLFL	9	HCV			719	24				1.4
2.0169	MYVGGVEHRL	10	HCV			633	24				0.026
2.0170	EYVLLFL	10	HCV			719	24				0.010
1.0139	SVPAEILRK	9	HCV		LORF	2249	3.11		0.016	0.07	
1.0955	QUTPSRR	9	HCV		ENV1	290	3.11		0.75	0.033	
1.0090	RLGVRATRK	9	HCV		CORE	43	3.11		0.74	0.16	
1.0123	HLFCHSKK	9	HCV		LORF	1391	3.11		0.54	0.19	
1.0122	HLFCHSKK	9	HCV		LORF	1390	3.11		0.25	0.010	
1.0952	KTSERSOPR	9	HCV		CORE	51	3.11		0.16	0.064	
1.0120	AVCTRGVAK	9	HCV		LORF	1183	3.11		0.016	0.038	
1.0143	EYFCVQPEK	9	HCV		LORF	2563	3.11		0.0019	0.033	
1.0137	ITRVESENK	9	HCV		LORF	2241	3.11		0.015	0.0099	
1.0957	QUTSLTGR	9	HCV		LORF	1042	3.11		0.0085	0.011	
1.0496	GVAGALVAFK	10	HCV		LORF	1858	3.11		0.87	1.1	
1.0480	HLHAPTCGCK	10	HCV		LORF	1227	3.11		0.57	0.0351	
1.1062	RMVVGVEHR	10	HCV		NS1/ENV2	632	3.11		0.27	0.012	
1.0485	HLFCHSKK	10	HCV		LORF	1390	3.11		0.27	0.025	
1.0484	TLGRCAYMSK	10	HCV		LORF	1261	3.11		0.17	0.13	
1.1067	GVGYLLFNR	10	HCV		LORF	3002	3.11		0.0029	0.032	
1.1063	LLFLLADAR	10	HCV		NS1/ENV2	723	3.11		0.015	0	

Table 23(e)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0014	FRDYVDRFY	9	HIV		CAC	298	1	0.090			
2.0129	NYQYMDOLY	9	HIV			875	1	0.064			
1.0028	TVLDVCDAY	9	HIV		POL	802	1	0.018			
1.0412	VTYLDVCDAY	10	HIV		POL	801	1	0.29	0	0.0004	
1.0415	VNYQYMDOLY	10	HIV		POL	874	1	0.25	0.0007	0.0090	
2.0252	VTYLDVCDAY	10	HIV			801	1	0.068			
1.0431	EYNYVDSQY	10	HIV		POL	1187	1	0.053			
1.0441	LVAVHVASGY	10	HIV		POL	1329	1	0.039			
1.0442	PAETCQETAY	10	HIV		POL	1345	1	0.013			
2.0251	ISKGFENPY	10	HIV			742	1	0.013			
2.0255	QMAVFHNFYK	10	HIV			1432	3		0.61	0.64	
2.0064	KYLKDOQLL	9	HIV			2778	24				0.76
2.0134	KYLKDOQLL	9	HIV			2778	24				0.32
2.0065	TYQYQEPF	9	HIV			1.033	24				0.30
2.0131	TYQYQEPF	9	HIV			1.033	24				0.30
2.0063	TYQEPFNL	9	HIV			1.036	24				0.052
2.0132	TYQEPFNL	9	HIV			1.036	24				0.033
2.0066	NYQYMDOLY	9	HIV			875	24				0.013
2.0207	NYKRWLCL	10	HIV			266	24				0.017
2.0190	NYKRWLCL	10	HIV			266	24				0.014
2.0209	LYFLASLJSL	10	HIV			506	24				0.014
1.0069	KLACRWPFYK	9	HIV		POL	1358	3.11		2.7	0.060	
1.0944	AVRHNFPRK	9	HIV		POL	1434	3.11		0.17	1.8	
1.0032	AHQSEMTK	9	HIV		POL	853	3.11		1.1	0.96	
1.0046	IVRWCKTPK	9	HIV		POL	1075	3.11		0.085	0.37	
1.0079	KLTEDRWVK	9	HIV		VIF	1712	3.11		0.013	0.27	
1.0027	GIHPFAGLK	9	HIV		POL	788	3.11		0.23	0.065	
1.0059	QIBQLKK	9	HIV		POL	1215	3.11		0.0091	0.16	
1.0039	KIWFPSYKGR	9	HIV		CAC	443	3.11		0.12	0.0005	
1.0072	ILATDQTK	9	HIV		POL	1458	3.11		0.025	0.098	
1.0036	MGVELHPDK	9	HIV		POL	925	3.11		0.064	0.086	
1.0062	YLAWVPAHK	9	HIV		POL	1227	3.11		0.077	0.057	
1.0038	KIWFPSYKGR	9	HIV		CAC	443	3.11		0.077	<0.0005	
1.0047	FVNTFFLVK	9	HIV		POL	1111	3.11		0.012	0.066	
1.0024	NTVFAIKK	9	HIV		POL	752	3.11		0.033	0.060	
1.0080	TVQCTHCK	9	HIV		ENV	2420	3.11		0.0021	0.046	
1.0013	ILDIHQCPK	9	HIV		CAC	287	3.11		0.042	0.0048	
1.0015	RDYVDRPYK	9	HIV		CAC	299	3.11		0.0007	0.040	
1.0058	GIHQAPDK	9	HIV		POL	1199	3.11		<0.0009	0.040	
1.0064	VLFDCIDK	9	HIV		POL	1254	3.11		0.038	0.032	
1.0026	LVDRELNK	9	HIV		POL	769	3.11		0.071	0.030	
1.0078	KVVFPRKAK	9	HIV		POL	1513	3.11		0.029	0.0039	
1.0942	MTKLEPRK	9	HIV		POL	859	3.11		<0.0008	0.016	
1.0463	TVYGVYVWK	10	HIV		ENV	2185	3.11		3.8	7.8	
1.0418	TVQPTVLEK	10	HIV		POL	935	3.11		0.16	5.6	
1.0447	AVRHNFPRK	10	HIV		POL	1434	3.11		0.46	0.85	
1.0437	KVLFDCIDK	10	HIV		POL	1253	3.11		0.36	0.78	
1.0408	KLVDFRELNK	10	HIV		POL	768	3.11		0.51	0.090	
1.0403	KLIQCMDCPK	10	HIV		POL	706	3.11		0.39	0.076	
1.0095	FLCKIWFYK	10	HIV		CAC	440	3.11		0.32	0.024	
1.1056	KIQNFVYYR	10	HIV		POL	1474	3.11		0.032	0.21	
1.0410	GIHPFAGLK	10	HIV		POL	788	3.11		0.071	0.17	
1.0026	LVLWYQLEK	10	HIV		POL	1117	3.11		0.056	0.082	
1.0098	MIGGICCFIK	10	HIV		POL	642	3.11		0.0099	0.055	
1.0413	MTKLEPRK	10	HIV		POL	859	3.11		0.075	0.038	
1.0453	VYQONSQIK	10	HIV		POL	1504	3.11		<0.0005	0.021	
1.0094	FLCKIWFYK	10	HIV		CAC	440	3.11		0.020	0.0013	
1.1059	IVQIQNLLR	10	HIV		ENV	2741	3.11		0.0024	0.019	
1.0417	FTTPDQGHQK	10	HIV		POL	909	3.11		<0.0002	0.075	
1.0405	LVECTEMEK	10	HIV		POL	729	3.11		0.0002	0.072	
1.0092	LVQANPDCK	10	HIV		CAC	327	3.11		<0.0002	0.071	

Table 23(f)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
1.0225	ISEYRHYCY	9	HPV	16	E6	80	1	7.8	0.0011	0.036	
1.0230	QAEFDRAHY	9	HPV	16	E7	44	1	0.021			
1.0610	LQDIETCVY	10	HPV	18	E6	25	1	0.25	0.0064	0.012	
2.0159	YSKISEYRHY	10	HPV	16	E6	77	1	0.17	<0.0009	0	
2.0162	YSKISEYRHY	10	HPV	16	E6	77	1	0.11	<0.0009	0	
1.0599	HGDTPTLHEY	10	HPV	16	E7	2	1	0.087			
1.0601	QFETTDLYCY	10	HPV	16	E7	16	1	0.033			
1.0913	IHDILECYV	10	HPV	16	E6	30	1	0.032			
2.0160	YSRIRLRHY	10	HPV	18	E6	72	1	0.018			
2.0164	YSRIRLRHY	10	HPV	18	E6	72	1	0.012			
1.0594	AVCDKCLQFY	10	HPV	16	E6	68	1	0.0095			
2.0161	LLIRCLROCK	10	HPV	18	E6	101	3		0.081	0.078	
2.0032	HTMILCMCK	9	HPV	18	E7	59	11		0.020	0.079	
2.0029	VYKTVLEL	9	HPV	18	E6	33	24				0.33
2.0027	CYSLYGTL	9	HPV	16	E6	87	24				0.057
2.0024	VYDFARDL	9	HPV	16	E6	69	24				0.002
2.0001	LYNLLIRCL	9	HPV	18	E6	98	24				0.019
2.0000	VYGDLEKL	9	HPV	18	E6	85	24				0.010
1.0239	SVYCDLEK	9	HPV	18	E6	84	3.11		0.39	2.3	
1.0243	SVYCDLEK	9	HPV	18	E6	84	3.11		0.53	1.1	
1.0244	SVYCDLEK	9	HPV	18	E6	84	3.11		0.70	0.95	
1.0226	TTLEQQYNK	9	HPV	16	E6	93	3.11		0.010	0.67	
1.0241	SIPHAACHK	9	HPV	18	E6	59	3.11		0.0094	0.25	
1.0237	SIPHAACHK	9	HPV	18	E6	59	3.11		0.017	0.12	
1.0233	IVCPICQK	9	HPV	16	E7	89	3.11		0.035	0.023	
1.0997	KLRHLNEQR	9	HPV	18	E6	117	3.11		0.025	<0.0005	
1.0234	LIRCLROCK	9	HPV	18	E6	102	3.11		0.019	0.012	
1.0853	IILECYCK	9	HPV	16	E6	33	3.11		0.0016	0.019	
1.0999	CIDFYSRIK	9	HPV	18	E6	68	3.11		0.017	0.0018	
1.0998	CIDFYSRIK	9	HPV	18	E6	68	3.11		0.010	0.0009	
1.0596	GTTLEQQYNK	10	HPV	16	E6	92	3.11		0.010	0.98	
1.0606	LLIRCLROCK	10	HPV	18	E6	101	3.11		0.076	0.29	
1.0598	LLIRCLROCK	10	HPV	16	E6	106	3.11		0.12	0.34	
1.0629	LLIRCLROCK	10	HPV	18	E6	101	3.11		0.16	0.11	
1.0614	LTEVFEFARK	10	HPV	18	E6	41	3.11		0.0009	0.31	
1.0605	GIVCPICQK	10	HPV	16	E7	88	3.11		0.0017	0.060	
1.0625	LTEVFEFARK	10	HPV	18	E6	41	3.11		0.0012	0.041	
1.0591	DILECVYCK	10	HPV	16	E6	32	3.11		0.0065	0.021	
1.1101	KLRHLNEQR	10	HPV	18	E6	117	3.11		0.013	0	
1.1095	CVYCKQQLR	10	HPV	16	E6	37	3.11		0.011	0.0059	

Table 23(9)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
2.0020	EVDPIGHLY	9	MAGE	3		161	1	18	0.0002	0.0009	
3.0172	EADPTSNLY	9	MAGE	5/51		161	1	9.9	0.0006	0.0006	0
1.0258	TQDLVQEKY	9	MAGE	1		240	1	2.1	0	0.0002	
3.0173	EVDPIGHVY	9	MAGE	6		161	1	1.9	<0.0002	<0.0002	0
1.0254	EADPTCHSY	9	MAGE	1		161	1	1.1	0	0	
1.0259	LVQEKYLEY	9	MAGE	1		243	1	0.42	0.0013	0.053	
6.0053	TSYVKYLEY	9	MAGE	1	new	275	1	0.099			
2.0009	SLPTTMINY	9	MAGE	3		9	1	0.053			
2.0011	GSVVGHWQY	9	MAGE	3		77	1	0.050			
2.0008	SSPTTINY	9	MAGE	2		9	1	0.043			
1.0252	MLESVIKLY	9	MAGE	1		128	1	0.011			
2.0147	ASLPTTMINY	10	MAGE	3		8	1	2.6	<0.0009	0.033	
2.0167	LTQDLVQEKY	10	MAGE	1		239	1	1.2	<0.0009	0.0073	
6.0114	ETSYVKYLEY	10	MAGE	1	new	274	1	0.56			
2.0141	ASSPTTINY	10	MAGE	2		8	1	0.17	<0.0009	0.026	
1.0648	DLVQEKYLEY	10	MAGE	1		242	1	0.044			
6.0065	TSYVKYLEY	9	MAGE	1	new	275	3		0.71	0.010	
4.0119	TTINPTQR	9	MAGE	1		66	3		0.043	0.37	
6.0064	ALAETSYVK	9	MAGE	1	new	271	3		0.31	0.36	
4.0132	LTQDLVQEK	9	MAGE	1		239	3		<0.0009	0.14	
6.0062	LVQEKYLEY	9	MAGE	1	new	243	3		0.0006	0.034	
4.0131	HSAYCEPRK	9	MAGE	1		229	3		0.014	0.0009	
4.0122	LFRAVITKK	9	MAGE	1		97	3		0.011	0.0005	
6.0124	RVRFFPSLR	10	MAGE	1	new	250	3		0.43	0.0099	
4.0161	ADLVGFLLLK	10	MAGE	1		107	3		0.35	0.29	
4.0160	ESLRAVITK	10	MAGE	1		95	3		0.14	0.088	
6.0119	DLVQEKYLEY	10	MAGE	1	new	242	3		0.032	0.0051	
6.0123	YVYKVSARVR	10	MAGE	1	new	283	3		0.019	0.0009	
4.0168	LSVMEVYDCR	10	MAGE	1		218	3		<0.0009	0.012	
4.0163	KAEMLESVIK	10	MAGE	1		125	3		<0.0009	0.0077	
6.0125	RALAETSYVK	10	MAGE	1	new	270	11		0.18	0.24	
2.0010	NYPLWSQSY	9	MAGE	3		16	24				0.027
2.0165	NYKHCFPEP	10	MAGE	1		135	24				0.25
2.0151	LYFATCLCL	10	MAGE	3		115	24				0.048
6.0126	SVYKYLEYVI	10	MAGE	1	new	276	24				0.036
1.0248	SLRAVITK	9	MAGE	1		96	3,11		4.1	2.7	
1.1006	SVMEVYDCR	9	MAGE	1		219	3,11		0.0083	1.3	
1.1004	TTINPTQR	9	MAGE	1		66	3,11		0.016	1.0	
1.0257	LTQDLVQEK	9	MAGE	1		239	3,11		0.0002	0.38	
1.0634	SLRAVITKK	10	MAGE	1		96	3,11		1.2	0.98	
1.0647	LLTQDLVQEK	10	MAGE	1		238	3,11		0.0004	0.16	
1.0640	MLESVIKLYK	10	MAGE	1		128	3,11		0.14	0.027	
1.0644	LLGDNQDMFK	10	MAGE	1/3		182	3,11		0.020	0.011	
1.0630	SLGQRSLHCK	10	MAGE	1		2	3,11		0.015	0.015	

Table 23(h)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0281	CSDCTTHY	9	p53			226	1	29.5	0.0010	0.029	
1.0667	CTAKSVTCTY	10	p53			117	1	0.33	0.023	0.049	0
1.0672	RVEGNLRVEY	10	p53			196	1	0.022			
1.0278	RVRAMATYK	9	p53			156	3.11		1.5	0.73	
1.0276	CTYSPALNK	9	p53			124	3.11		0.46	1.1	
1.0285	NTSSSPQPK	9	p53			311	3.11		0.0039	0.095	
1.0284	KTEENLRK	9	p53			283	3.11		0.0015	0.091	
1.0287	ELNEALELK	9	p53			343	3.11		0.020	0.0052	
1.0678	RTEENLRKK	10	p53			283	3.11		3.3	0.0080	
1.1113	KTYQGSYCFR	10	p53			101	3.11		2.6	0.88	
1.1115	VVRRCFPIHER	10	p53			172	3.11		0.099	0.0017	
1.0679	NTSSSPQPKK	10	p53			311	3.11		0.0035	0.054	
1.1121	RVCACFGDR	10	p53			273	3.11		0.014	0.011	
1.1116	GLAPPOHLIR	10	p53			187	3.11		0.013	0.0006	

Table 23(i)

114

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
3.0175	KCEYPVEMY	9	PAP			322	1	3.4	<0.0002	0.0002	0
3.0174	LCGYRQRY	9	PAP			81	1	0.78	<0.0002	0.0002	0
3.0166	ASCHLTLY	9	PAP			311	1	0.77	<0.0002	0.055	0
3.0163	ESYKHEQVY	9	PAP			95	1	0.098	<0.0002	0.0002	0
3.0237	LSLSLSLY	10	PAP			238	1	14	0.0026	0.0004	0
3.0235	LSLSLSLY	10	PAP			238	1	12	0.0025	0.0004	0
3.0236	LTQLGMEQHY	10	PAP			70	1	0.62	0.015	0.0024	0.0022
3.0238	KCEYPVEMY	10	PAP			322	1	0.018			
3.0230	LVNEILNTHMK	10	PAP			263	3		0.056	0.12	
3.0158	ATQPSYK	9	PAP			274	11		0.10	1.2	
3.0231	ETLKSEERK	10	PAP			170	11		<0.0004	0.014	
3.0161	LYFEKGEYF	9	PAP			318	24				2.5
3.0160	LYCESVHNF	9	PAP			213	24				0.44
3.0159	PYKDFATL	9	PAP			183	24				0.11
3.0162	VYNGLLPFY	9	PAP			302	24				0.032
3.0232	PYASCHLTSL	10	PAP			309	24				0.024

Table 23(j)

115

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0270	ALPERPSLY	9	PSA			231	1	0.011			
2.0157	VSHSPHPLY	10	PSA			88	1	0.15	<0.0003	0.0015	
1.0265	FLYDMSLLK	9	PSA			95	3.11		0.24	0.007	
1.0273	VVHYRKWIK	9	PSA			242	3.11		0.0072	0.093	
1.0272	YTKVVHYRK	9	PSA			239	3.11		0.0006	0.058	
1.1009	SLIGNRFLR	9	PSA			100	3.11		0.0024	0.047	
1.0260	IVCGWECEK	9	PSA			21	3.11		0.041	0.019	
1.0269	QVHPQKVTK	9	PSA			182	3.11		0.0040	0.014	
1.1112	SLYTKVVHYR	10	PSA			237	3.11		0.28	0.23	
1.0653	LTAAHQIRNK	10	PSA			57	3.11		0.14	0.093	
1.0651	RVCGWECEK	10	PSA			20	3.11		0.046	0.067	
1.0662	KVVHYRKWIK	10	PSA			241	3.11		0.045	0.045	
1.1111	VTKFMLCAGR	10	PSA			188	3.11		0.0003	0.012	
3.0108	MLLRLEPA	9	PSA			118	Random				

Table 23 (k)

SENT BY:

8- 6-93 : 15:27 :

CYTEL-

415 543 5043:#25/25

TABLE 24: CTL EPTOPES IDENTIFIED IN PEPTIDE SCREENING.

Sequence	Antigen	Motif	Id
EVDPIGHLY	MAGE3	A01	1044.07
ASSLEPTMNY	MAGE3	A01	1044.01
EADPTGHSY	MAGE1	A01	958.01
SSLEPTMNY*	MAGE3	A01	1072.02*
GSVVGNMQY*	MAGE3	A01	1072.03*
ALAETSYVK*	MAGEIN	A03	1072.38*
SLERAVITK	MAGE1	A11	1072.13
RALAETSYVK	MAGEIN	A11	1072.39
ESLEFRAVITK	MAGE1	A11	1072.15
KVYLAWPAAHK	HIV	A3/11*	1069.42*
TVYYGVPVWK	HIV	A03	1069.43
KLGRMPVK	HIV	A03	1069.44
KMIGTIGETIK	HIV	A03	1069.45
ALFQSSMTK	HIV	A03	966.01
WTYQIYOEPFK	HIV	A03	1069.46
ELGKIWP SHK*	HIV	A03	1069.56*
TVYYGVPVWK	HIV	A11	1052.03
VTVYYGVPVWK	HIV	A11	1069.47
GVAGALVAFK	HCV	A03	1073.10
CTCGSSDLY	HCV	A11	1069.62
GVAGALVAFK	HCV	A11	1052.05
LDDTASALY*	HBV	A01	1069.01*
TLWKAGILYK	HBV	A03	1069.15
* borderline positive			

Table 25 a
Peptides Synthesized
by Cytel For Loading
Onto Acid Stripped
Autologous PBMCs and
PHA Blasts

	Peptide ID #	Antigen	Sequence
	777.03	HBs	FLLTRILTI
10	924.07	HBc	FLPSDFFPSV
	927.32	HBp	GLYSSTVPV
	938.01	MAGE 1	EADPTGHSY
	939.03	PSA	VLVHPQWVL
	941.01	HBc	FLPSDYFPSV
15	1044.04	PAP	ILLWDPIPV
	1044.05	PSA	KLQCVDLVHI
	1044.06	PSA	MLLRLSEPAEL

20 Table 25 b
Cell Population

	¹²⁵ I-Labeled Peptide +/- Cold Peptide	CPMS +/- std. dev.	
	JY acid stripped - cold peptide	3553 ± 157	n = 3
25	JY acid stripped + cold peptide	13	n = 1
	JY control -cold peptide	370 ± 37	n = 3
	JY control + cold peptide	50	n = 1

WHAT IS CLAIMED IS:

1. A composition comprising an immunogenic peptide having a HLA-A3.2 binding motif, which immunogenic peptide has
5 between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E;

and a second conserved residue of K, R, Y, H and F;

10 wherein the first and second conserved residues are separated by 6 to 7 residues.

2. The composition of claim 1, wherein the first conserved residue is at the second position from the
15 N-terminus.

3. A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following
20 residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of D, E, A, S and T;

a third conserved residue of Y;

25 wherein the first and second conserved residues are adjacent and the second and third conserved residues are separated by 5 or 6 residues.

4. The composition of claim 3, wherein the first conserved residue is at the second position from the
30 N-terminus.

5. A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following
35 residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of Y;

wherein the first and second conserved residues are separated by 6 to 7 residues..

5 6. The composition of claim 5, wherein the first conserved residue is at the second position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

10 7. A composition comprising an immunogenic peptide having an HLA-A1 binding motif, which immunogenic peptide has between 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus.

 a first conserved residue of D, E, A, S and T; and
 a second conserved residue of Y;

15 wherein the first and second conserved residues are separated by 5 to 6 residues.

20 8. The composition of claim 5, wherein the first conserved residue is at the third position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

25 9. A composition comprising an immunogenic peptide having a HLA-A11 binding motif, which peptide has between about 9 and about 10 residues and the following residues, from the Nterminus to the C-terminus:

 a first conserved residue of L, M, I, V, A, S, T, G, N, Q, C, F, D, E; and

 a second conserved residue of K, R, H;

30 wherein the first and second conserved residues are separated by 6 to 7 residues.

35 10. The composition of claim 9, wherein the first conserved residue is at the second position from the N-terminus.

11. A composition comprising an immunogenic peptide having a HLA-A24.1 binding motif, which immunogenic peptide

has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of Y, F, W; and

a second conserved residue of F, I, L, W, M;

5 wherein the first and second conserved residues are separated by 6 to 7 residues.

12. The composition of claim 11, wherein the first conserved residue is at the second position from the
10 N-terminus.

13. A composition comprising an immunogenic peptide having an HLA-A3.2 binding motif, which immunogenic peptide has 9 or 10 residues:

15 a first conserved residue at the second position selected from the group consisting of A, I, L, M, T, and V; and a second conserved residue at the C terminal position selected from the group consisting of K and R.

20 wherein the first and second conserved residues are separated by 6 to 7 residues.

14. A composition comprising an immunogenic peptide having an HLA-A11 binding motif, which immunogenic peptide has 9 or 10 residues and the following residues, from the
25 N-terminus to the C-terminus:

a first conserved residue at the second position from the N terminus selected from the group consisting of A, I, L, M, T and V; and

30 a second conserved residue at the C terminal position selected from the group consisting of K;

wherein the first and second conserved residues are separated by 6 to 7 residues.

15. A pharmaceutical composition comprising a
35 pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A3.2 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E; and
a second conserved residue of K, R and Y;
wherein the first and second conserved residues are
5 separated by 6 to 7 residues.

16. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A1 binding motif, which immunogenic peptide has
10 between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and
a second conserved residue of D, E, A, S and T;
a third conserved residue of Y;

15 wherein the first and second conserved residues are
separated by 1 residue and the second and third conserved
residues are separated by 5 or 6 residues.

17. A pharmaceutical composition comprising a
20 pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A1 binding motif, which immunogenic peptide has
between about 9 and about 10 residues and the following
residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S or M; and
25 a second conserved residue of Tyr;

wherein the first and second conserved residues are
separated by 6 to 7 residues.

18. A pharmaceutical composition comprising a
30 pharmaceutically acceptable carrier and an immunogenic peptide
having a HLA-A1 binding motif, which peptide has between about
9 and about 10 residues and the following residues, from the
N-
terminus to the C-terminus:

35 a first conserved residue of D, E, S, T; and
a second conserved residue of Y;

wherein the first and second conserved residues are
separated by 5 to 6 residues.

19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A24.1 binding motif, which peptide has
a first conserved residue of Y, F, W; and
5 a second conserved residue of F, I, L, W, or m;
wherein the first and second conserved residues are separated by 6 to 7 residues.

20. A method of identifying an immunogenic peptide
10 comprising the following steps:
determining a binding motif for an MHC molecule encoded by a preselected MHC Class I allele;
screening an amino acid sequence of an antigenic protein for the presence of the binding motif;
15 selecting a sequence in the antigenic protein having the binding motif;
preparing a test peptide of about 8 and about 11 residues comprising the selected subsequences;
determining the ability of the test peptide to bind
20 to the preselected MHC allele and induce a CTL response, thereby identifying immunogenic peptides.

1/25

HLA-A PURIFICATION AND PEPTIDE ELUTION

Cellular Source: HLA-Antigens
($5-10 \times 10^6$ cell equivalents)

- a) EBV transformed B cell lines - homozygous
- b) HLA-A transfectants - e.g. .221-HLA-A1
- c) P815 transfectants (mouse mastocytoma)

↓
Detergent Lysis
(10^6 cells/ml)

1% NP-40 or 1% Renex 30 plus
protease inhibitors - 1 hr, 4°C

↓
Detergent Lysate

Centrifugation at 15,000xg,
30 min.

↓
Affinity Chromatography

mAb-Sepharose 5 mg/ml
5-10 ml column

↓
Purified HLA-A Antigen

Anticipated yields 450-900 µg

↓
Acid Treatment

10% acetic acid, 5 min, 100°C

↓
Peptides

YM3 filtration, 3kD cut-off

↓
Sequence/Motif

D. Hunt - HPLC/EI-TMS
Cytel - HPLC/ABI 477A

2/25

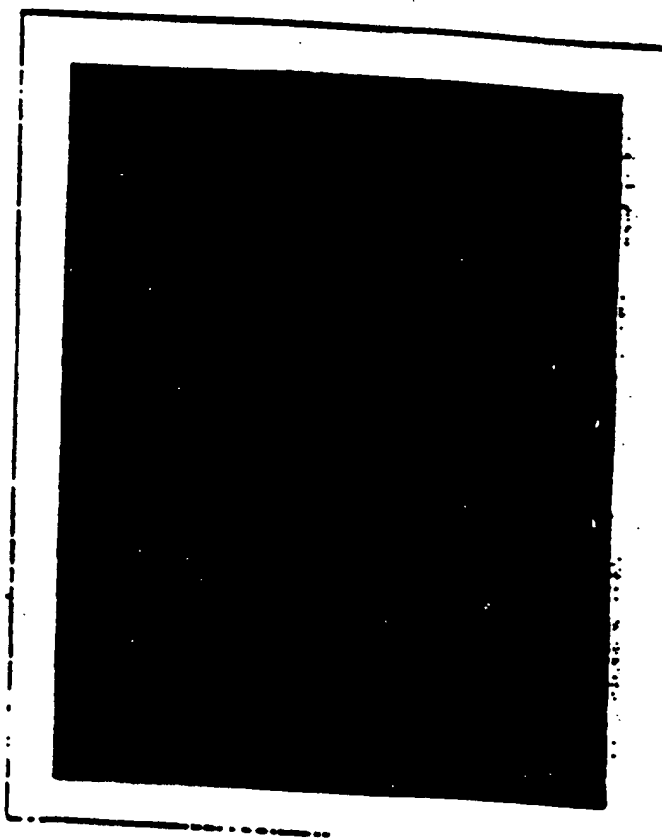


Figure 2

3/25

Fig. 3

0111 0 721
11021

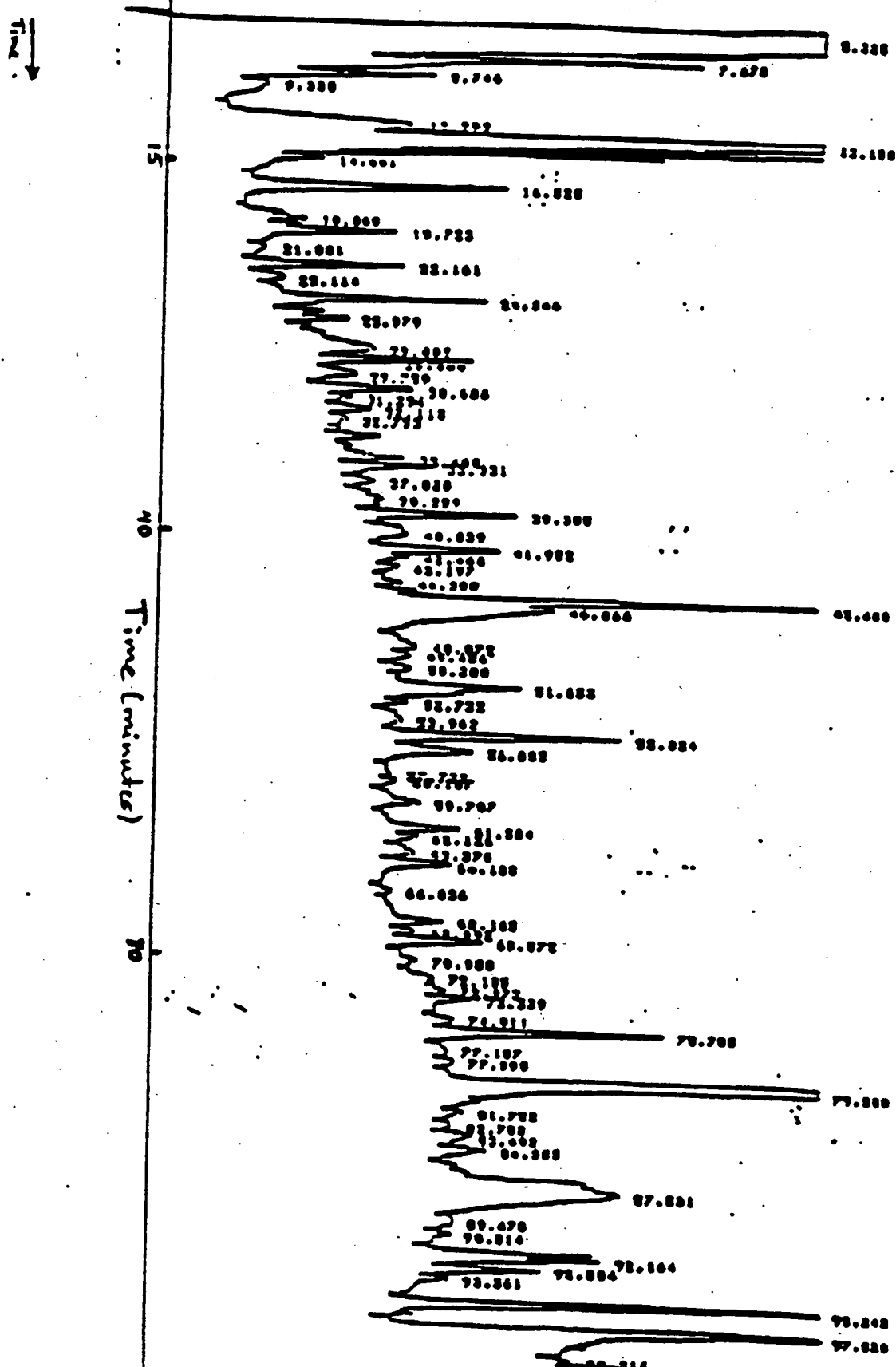
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17088

... 1. 1992 10000000
Absorbance Peptides from HLA A3
2.645 A214

2.645

A 214



4/25

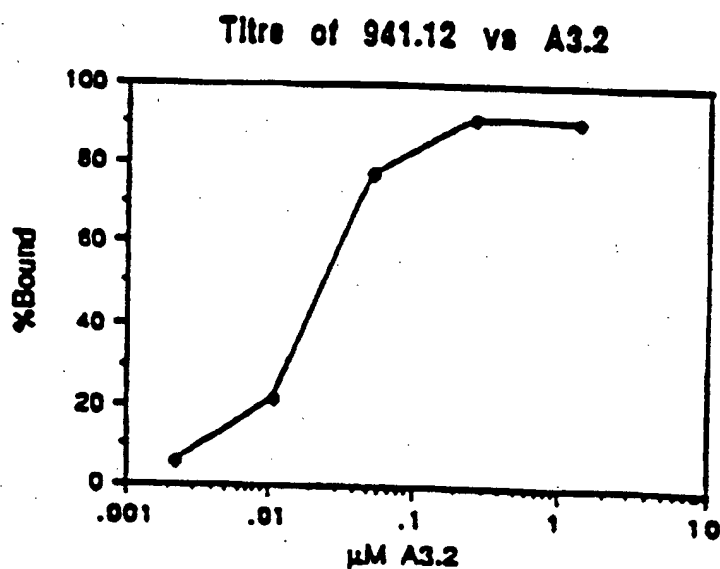


Figure 4

5/25

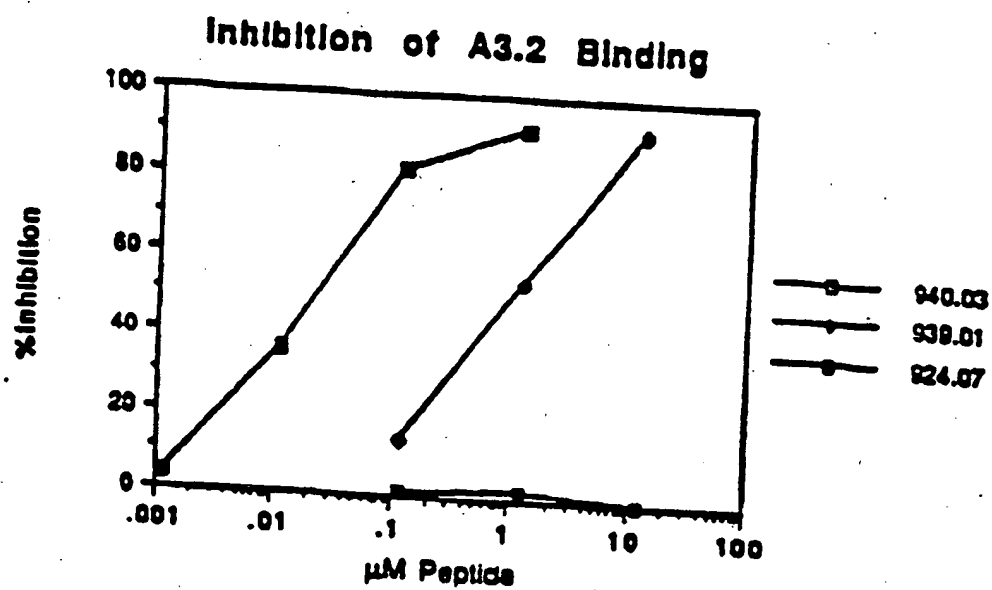


Figure 5

6/25

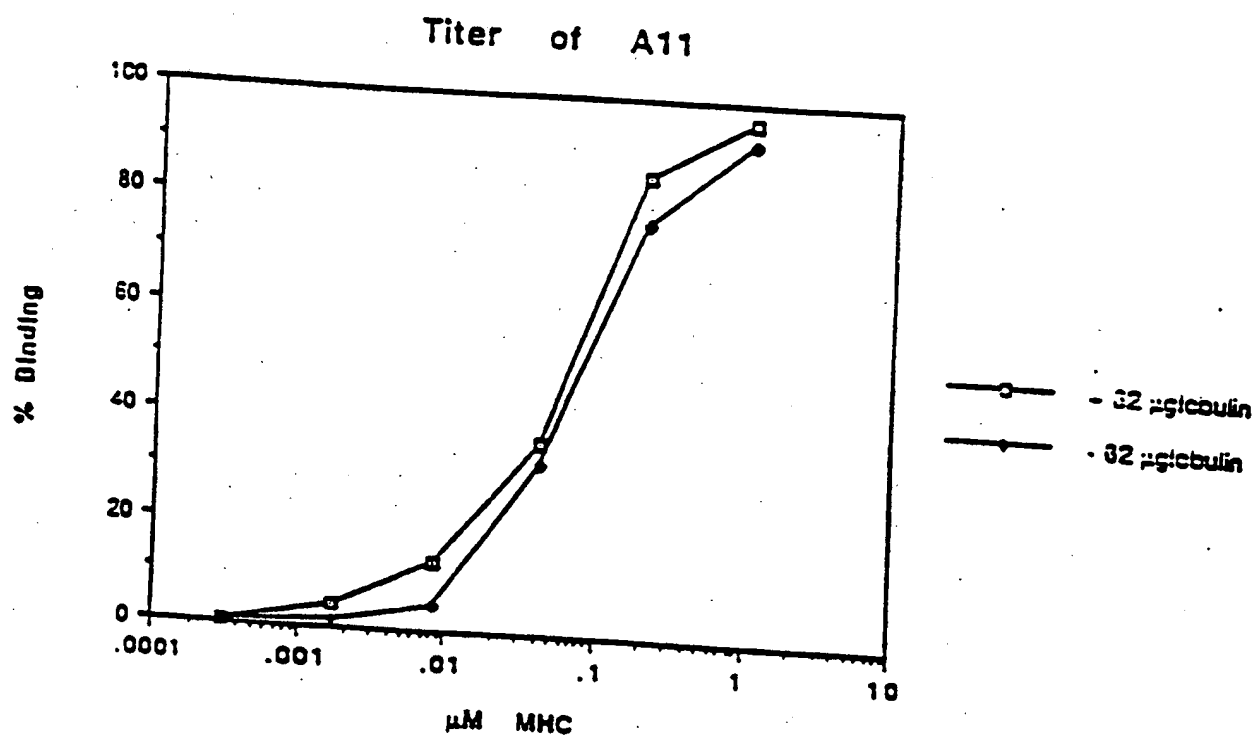


Figure 6

7/25

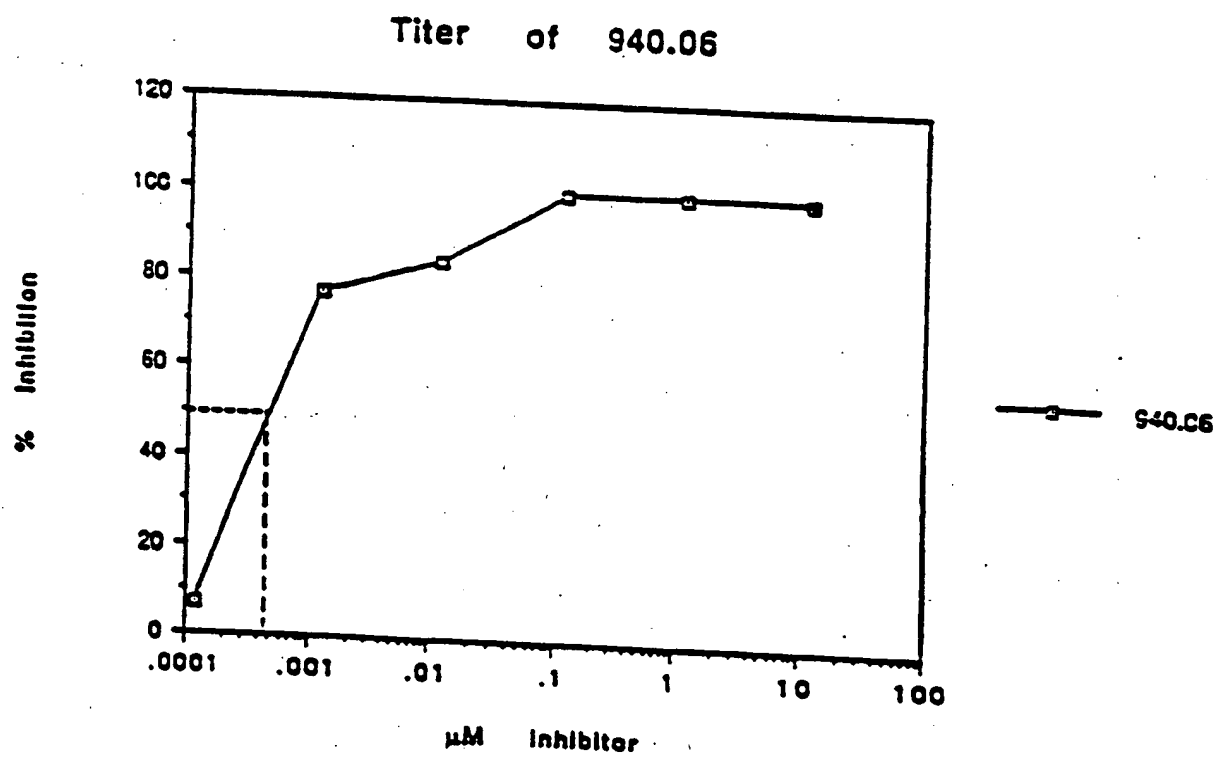


Figure 7

8/25

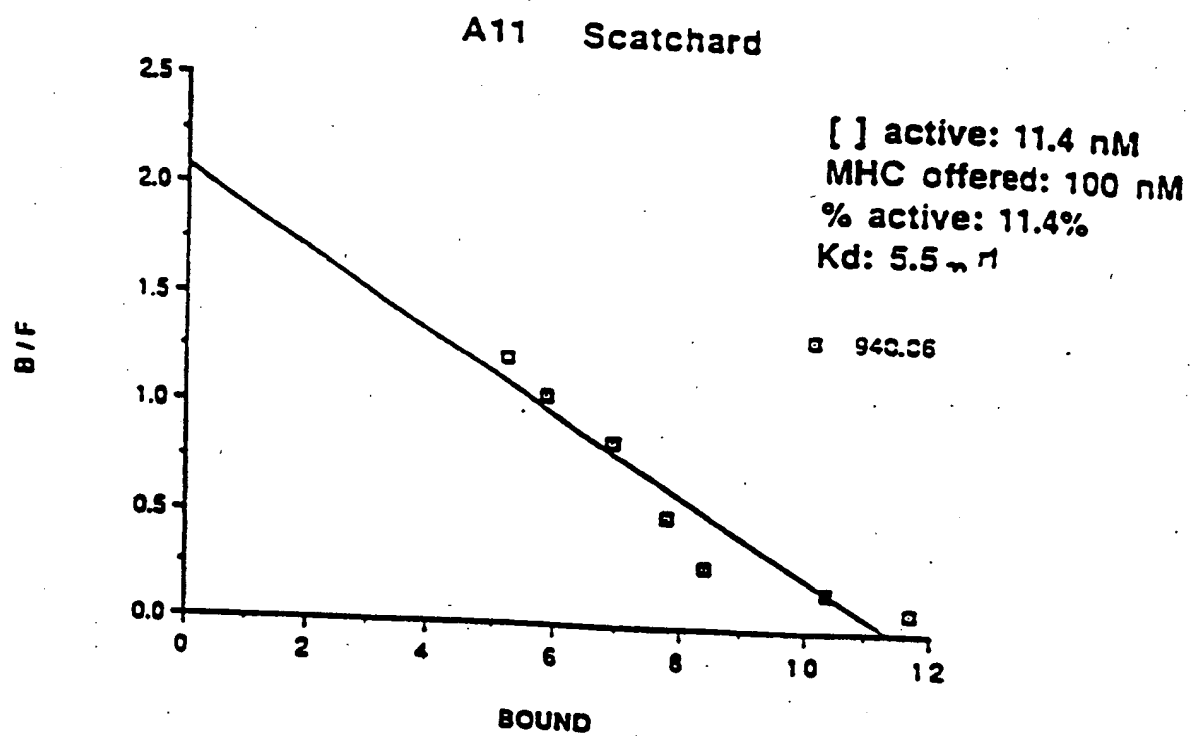


Figure 8

9/25

Titer of 944.02 against A1.

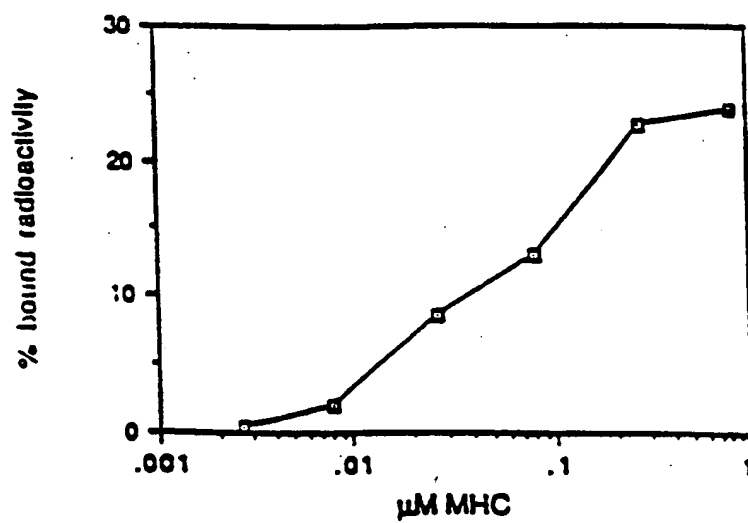


Figure 9

10/25

A1 inhibition titration.

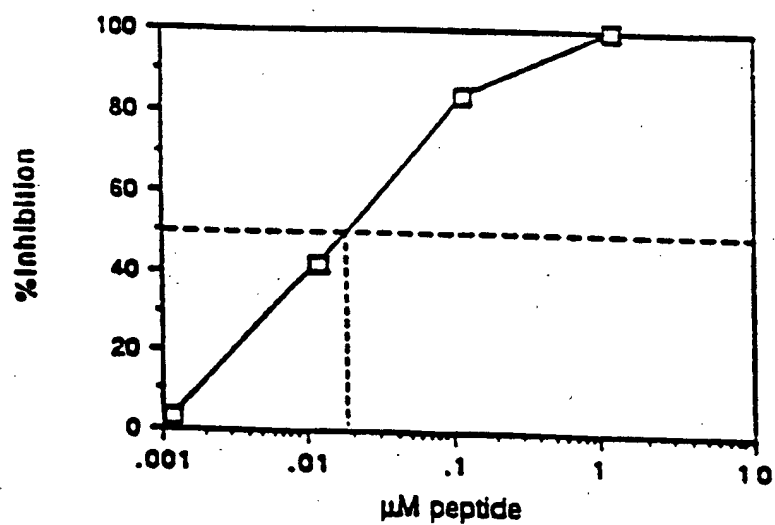


Figure 10

11/25

Scatchard for A1 vs. 944.02

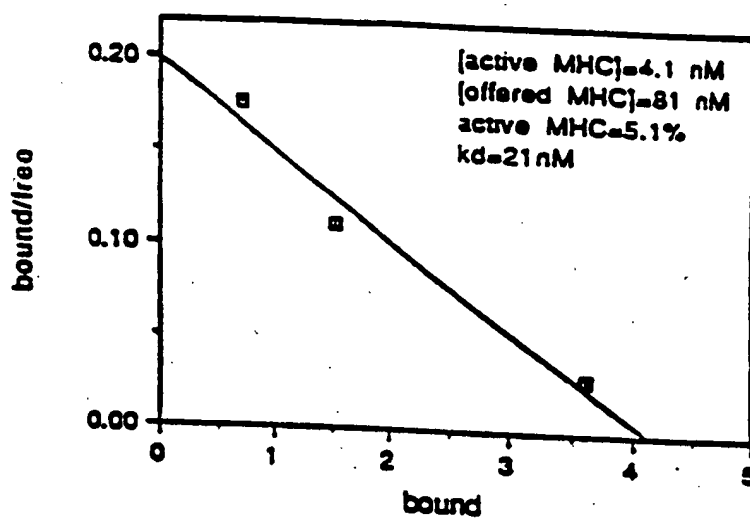


Figure 11

12/25

A24 titration.

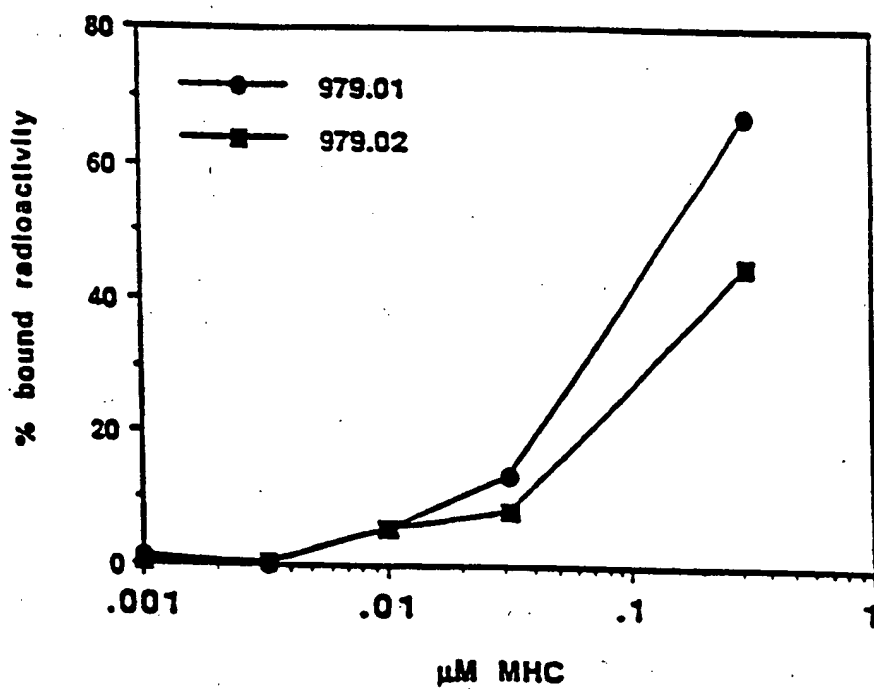


Figure 12

13/25

A24 Inhibition.

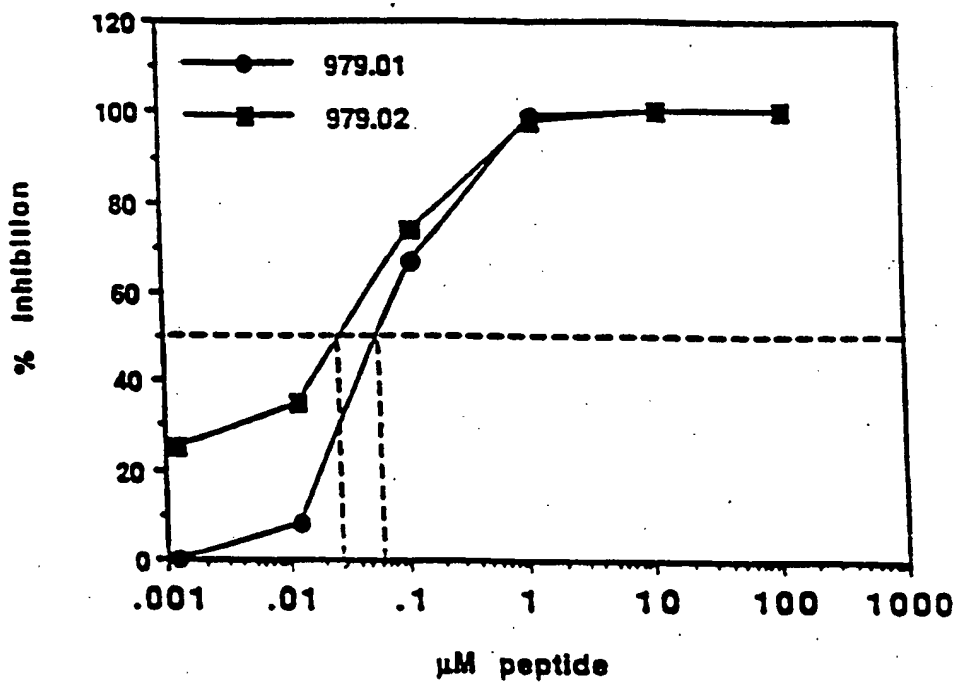


Figure 13

14/25

Figure 14A

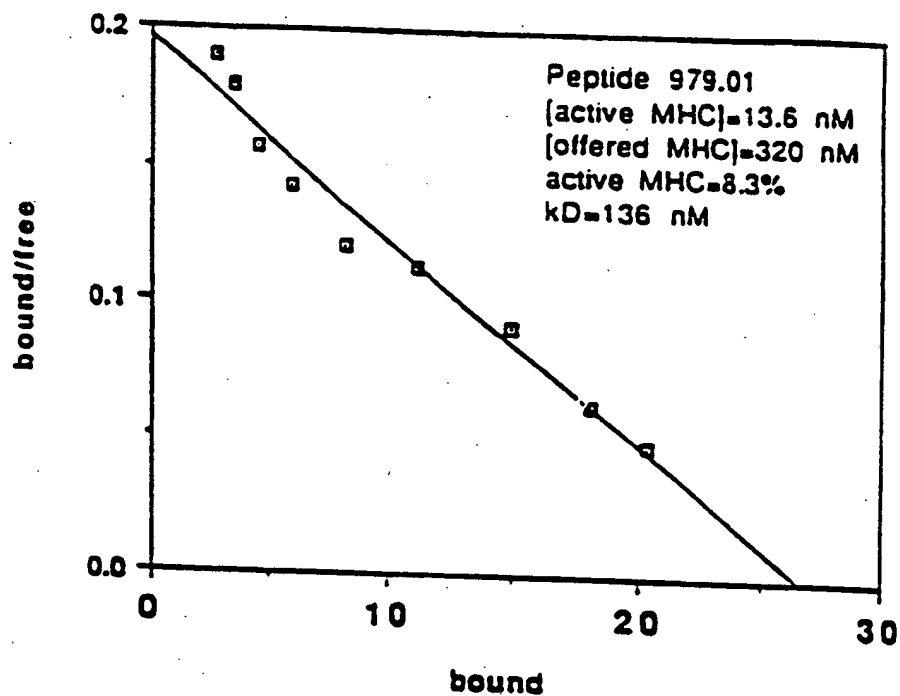
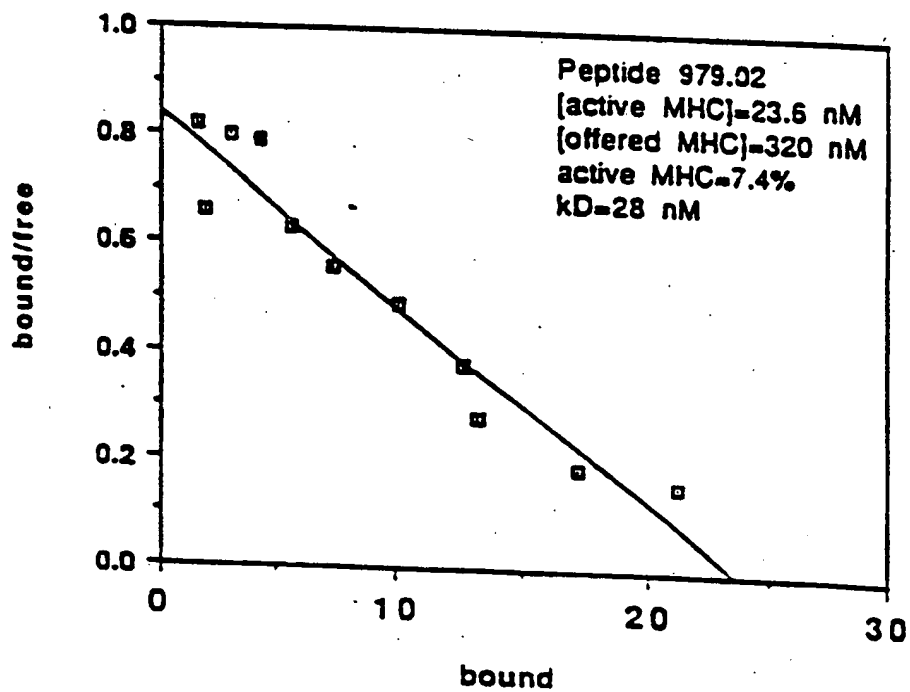
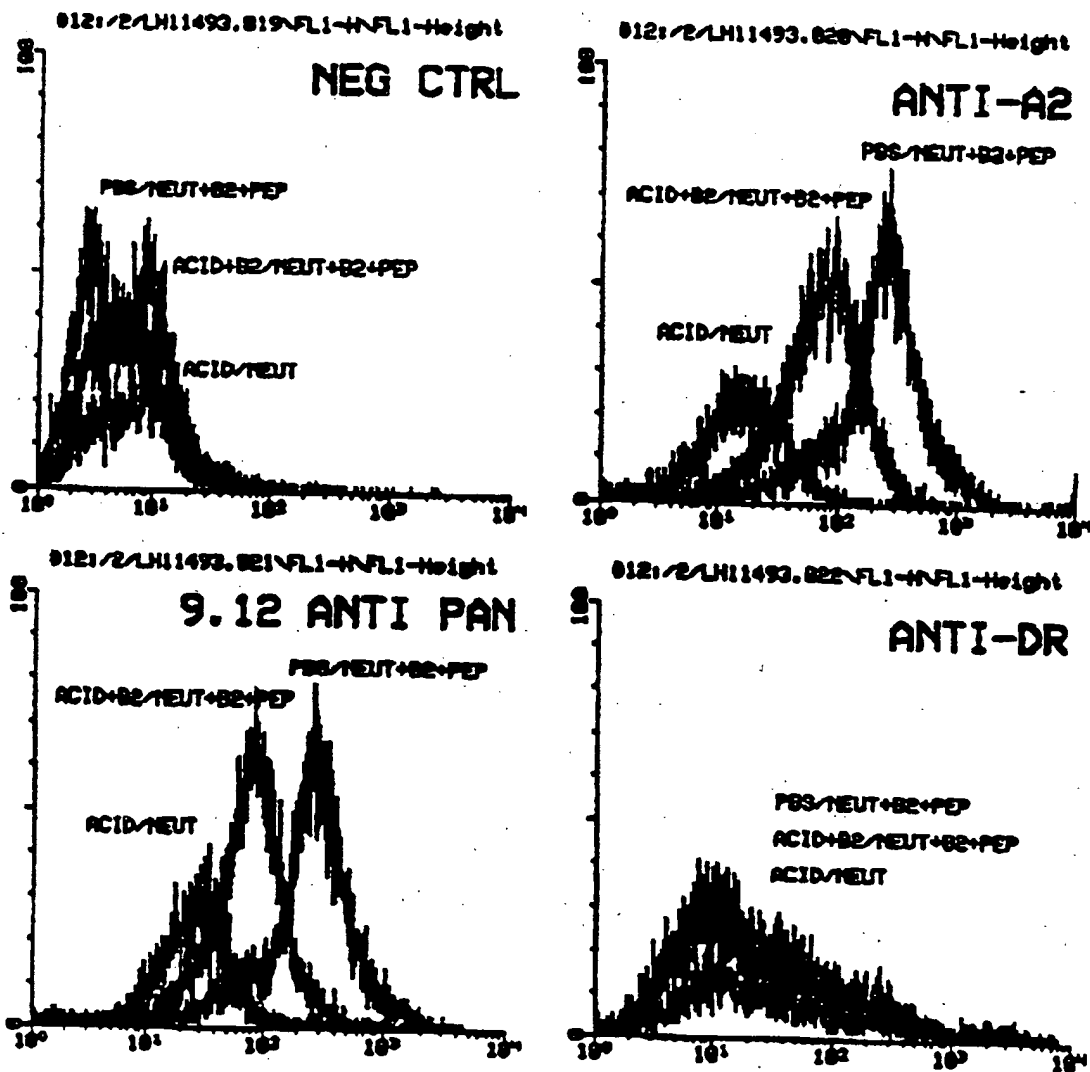


Figure 14B



15/25

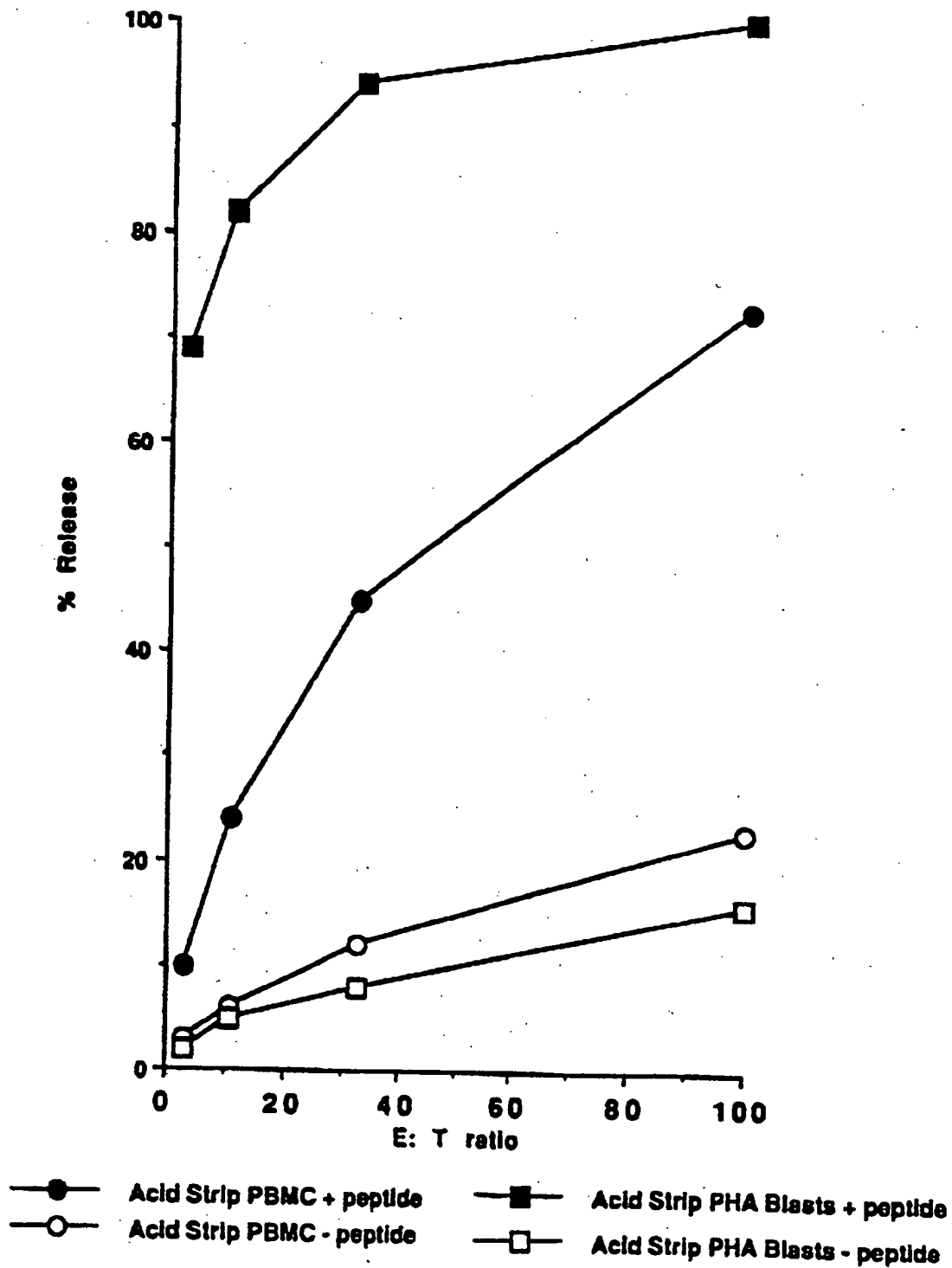
Figure 15



16/25

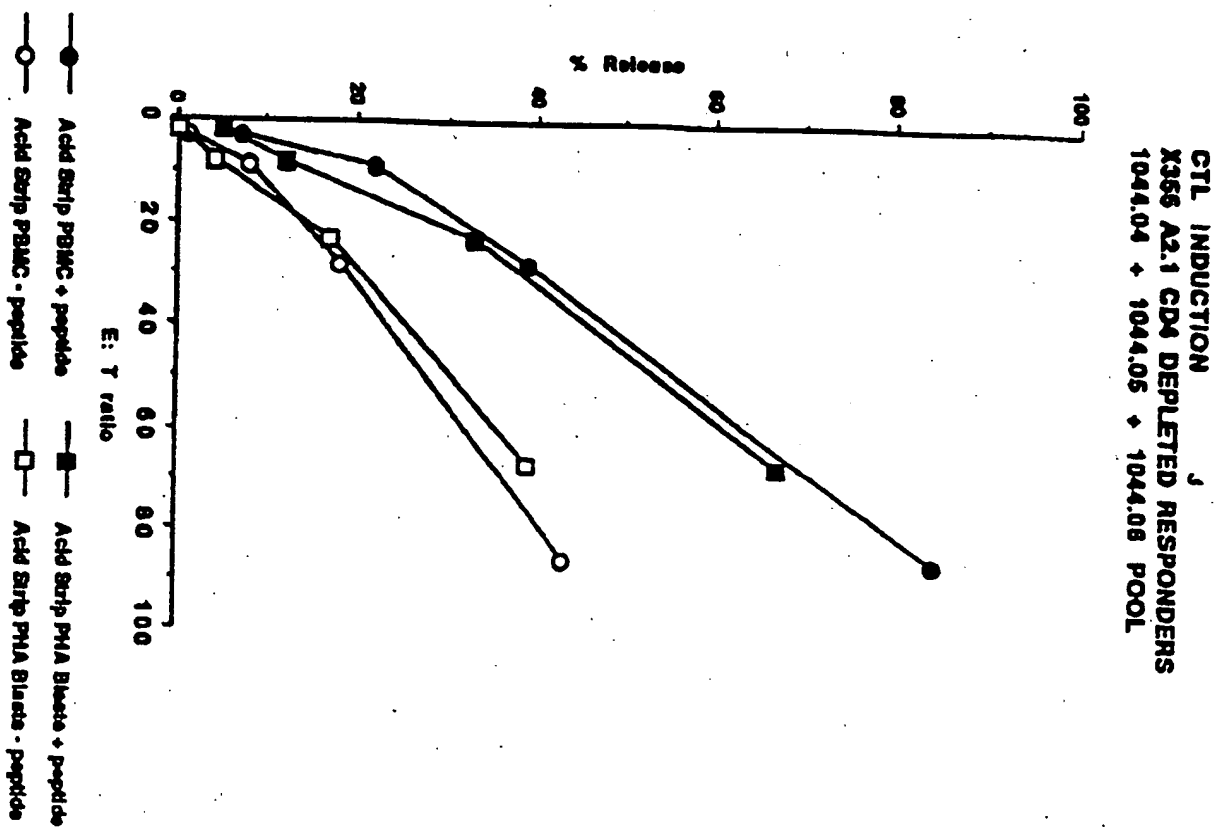
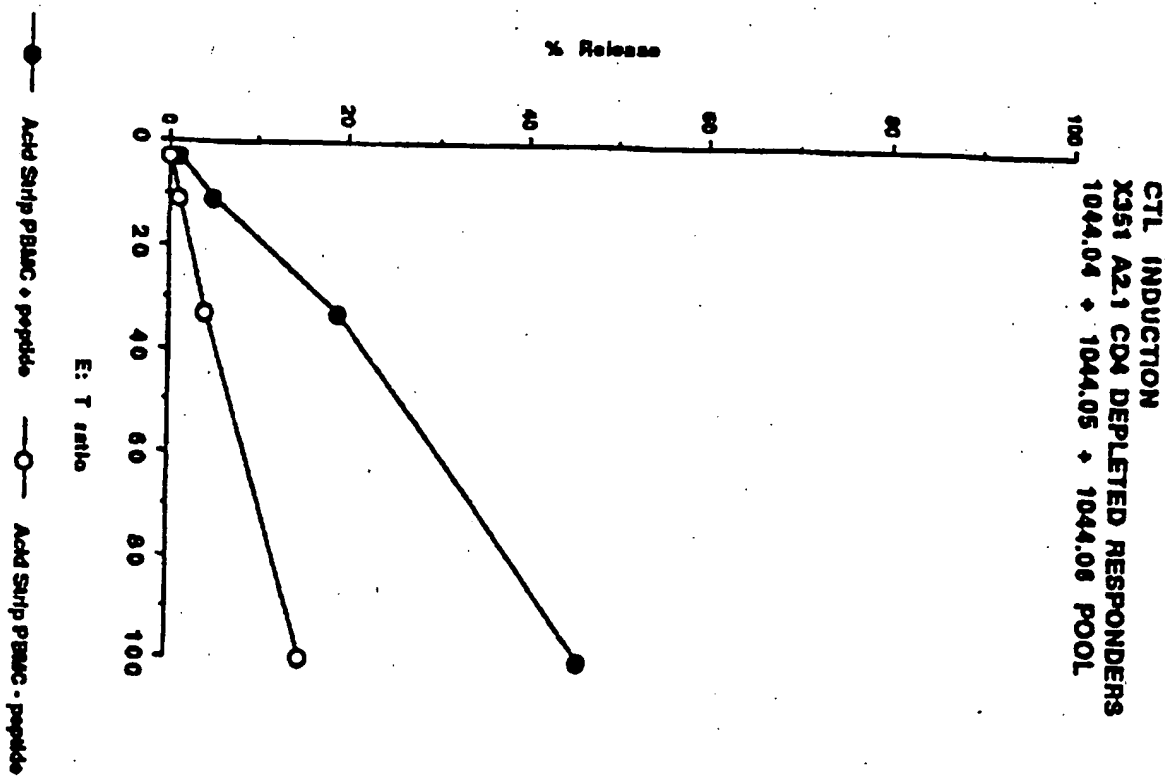
Figure 16

CTL INDUCTION
GC43 A2.1 CD4 DEPLETED RESPONDER
777.03 + 924.07 + 927.32 POOL



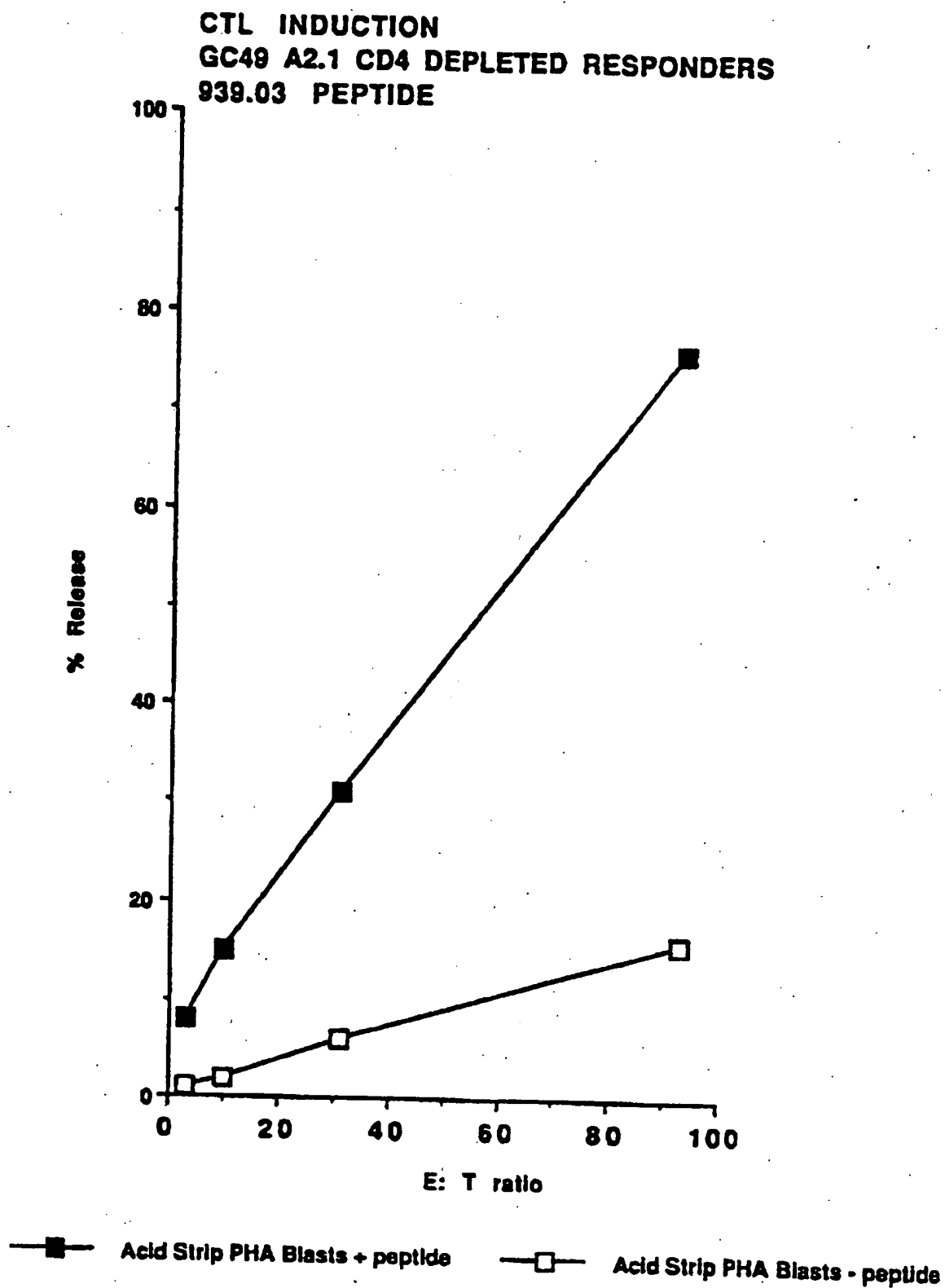
17/25

Figure 17



18/25

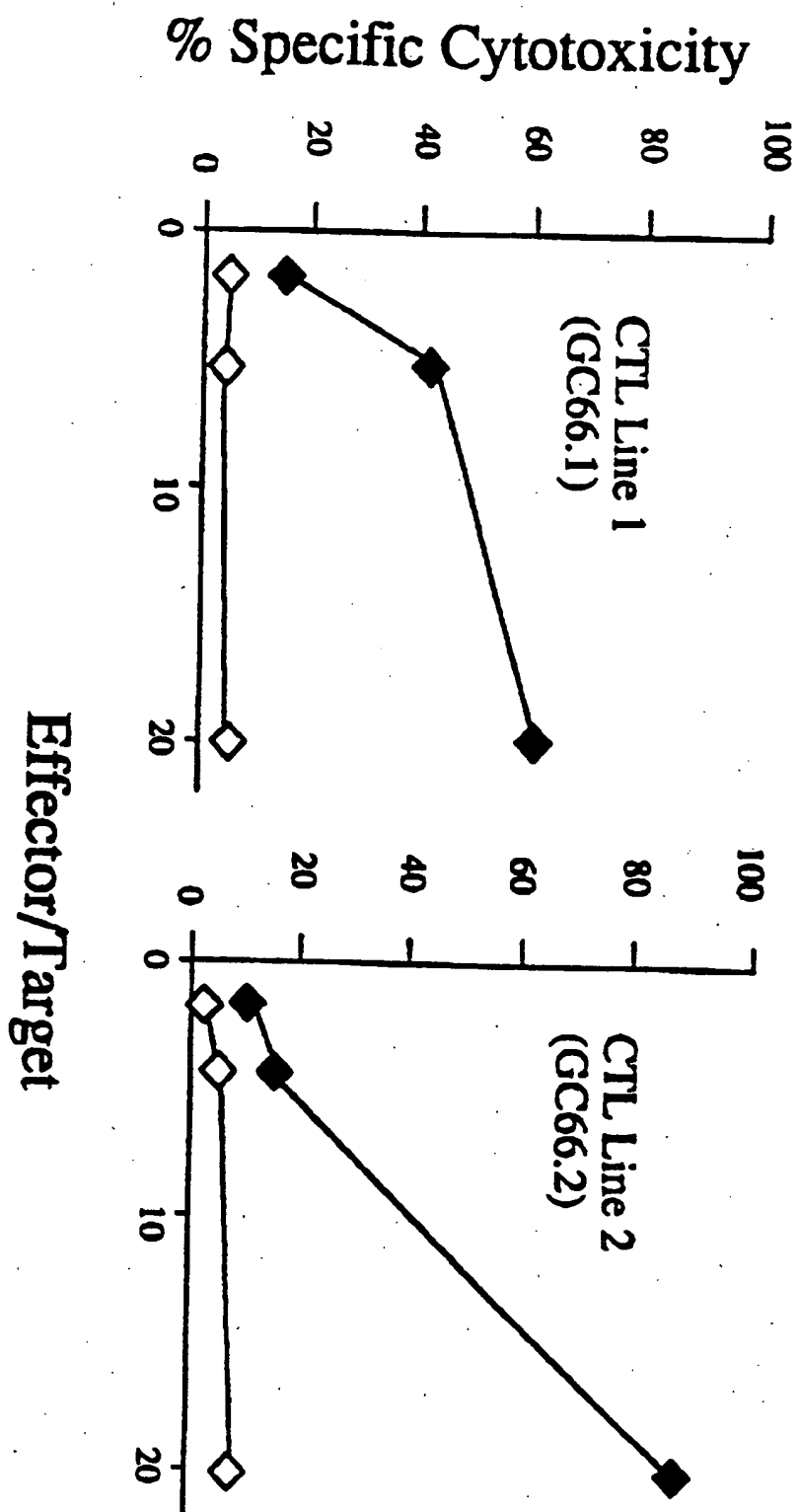
Figure 18



19/25

MAGE1 Peptide Specific, HLA-A1-Restricted CTL Can Kill Melanoma Cells Expressing the Endogenous Antigen

Figure 19



- ◆ 938-mel (HLA-A1/24, MAGE1⁺)
- ◇ 888-mel (HLA-A1/24, MAGE1⁻)

20/25

Figure 20

**Cytotoxic Activity of an HLA-A1-Restricted CTL Line
Specific for a MAGE3 Peptide (1044.07)**

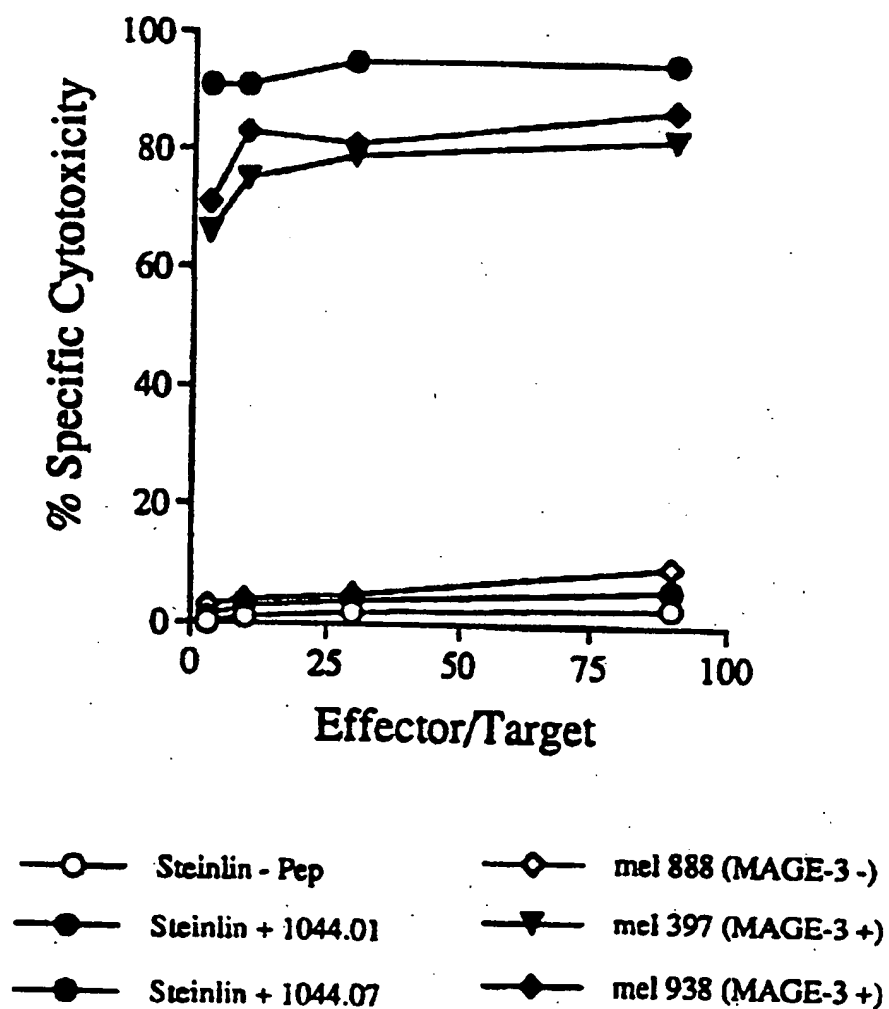
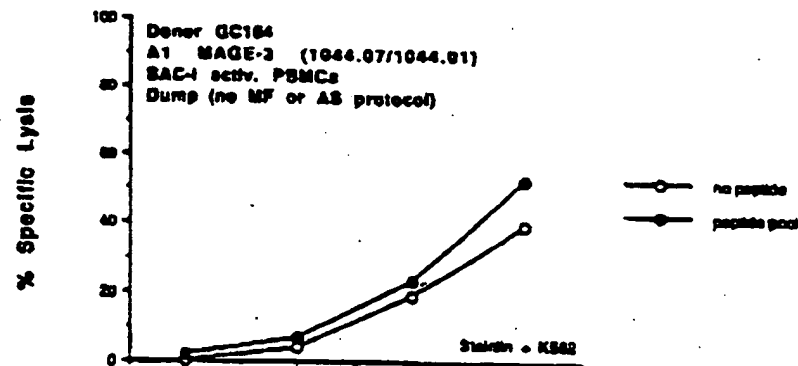
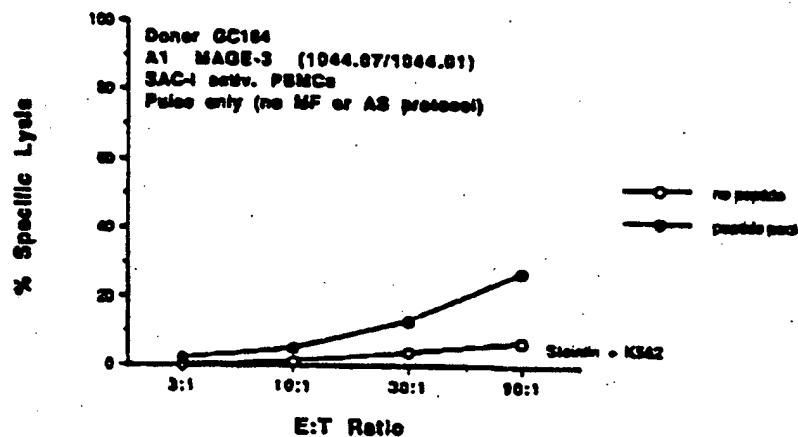
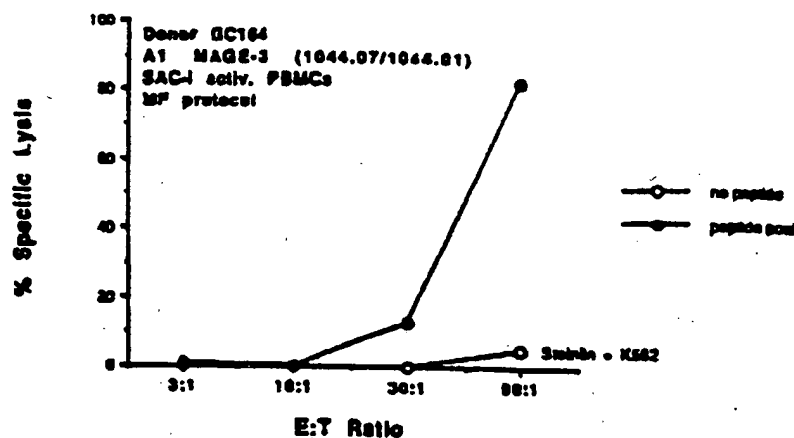
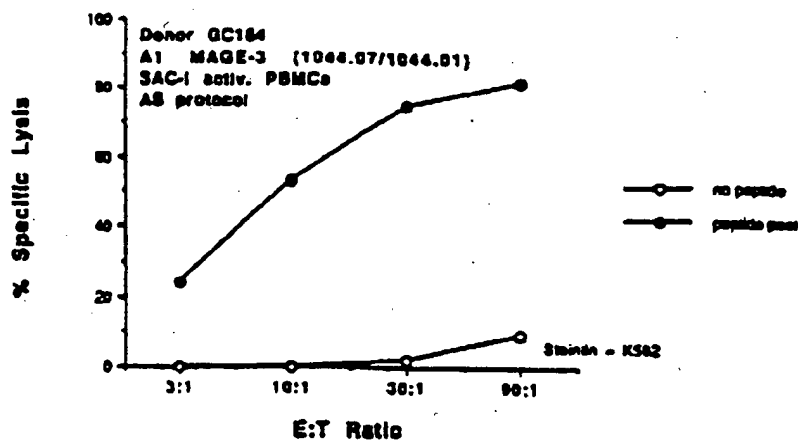


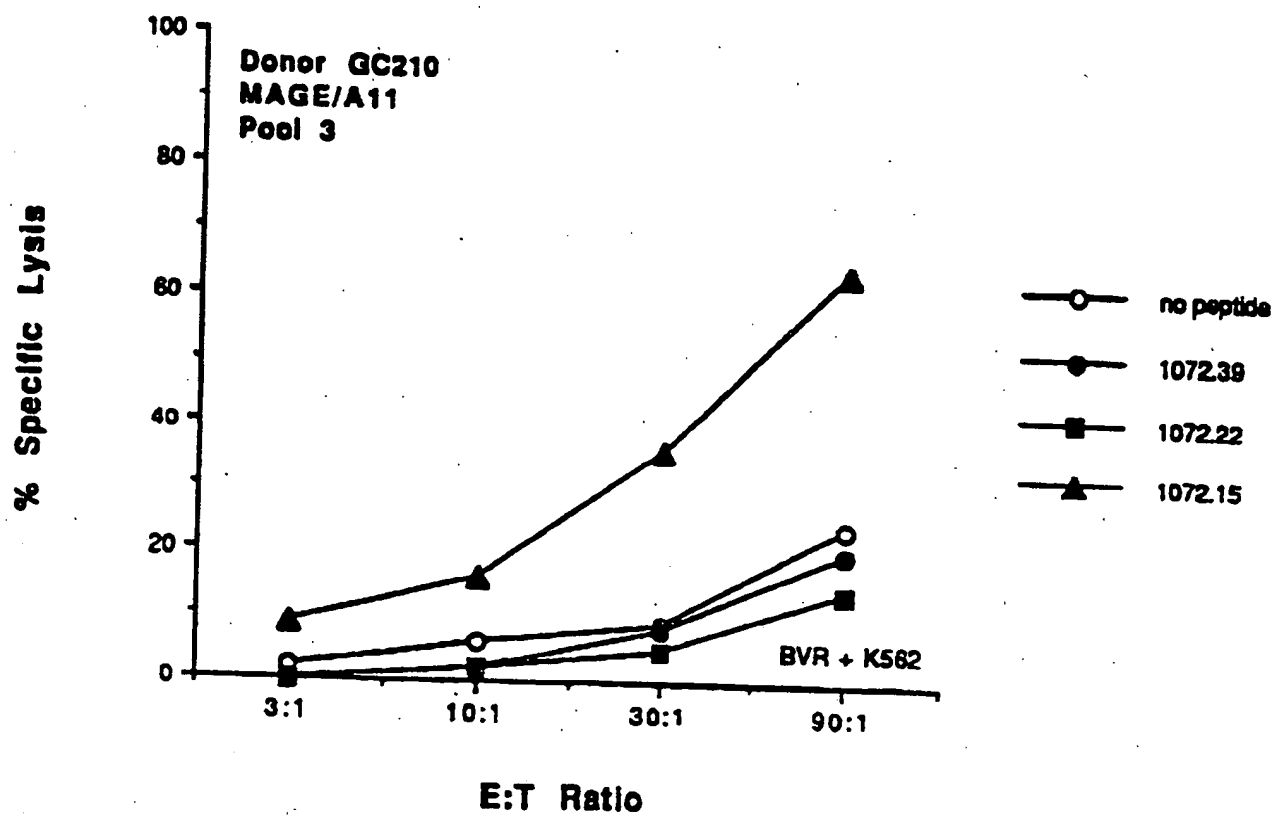
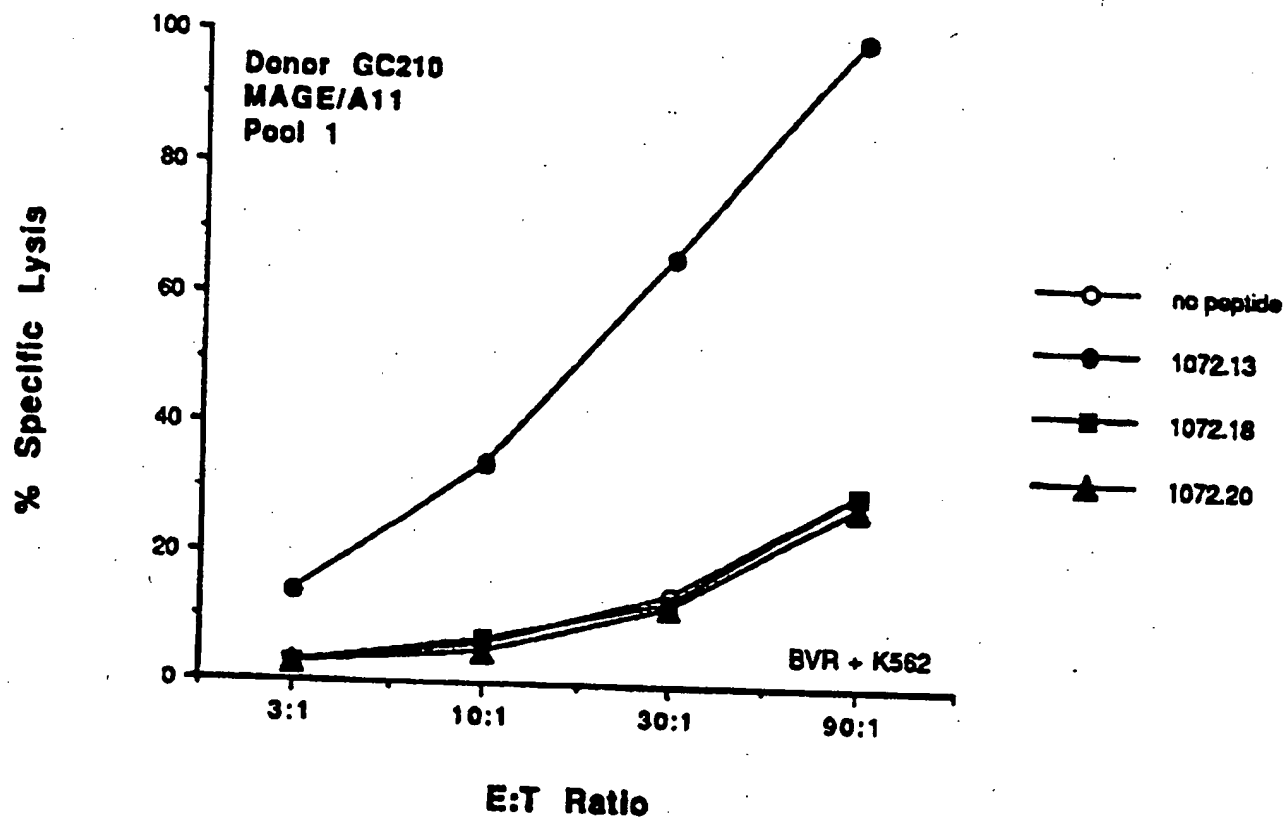
Fig. 21

Induction Using Different Methods to Load Peptides onto SAC-I Cells.



22/25

Figure 22
Peptide Screening:
MAGE/A11



23/25

Figure 23

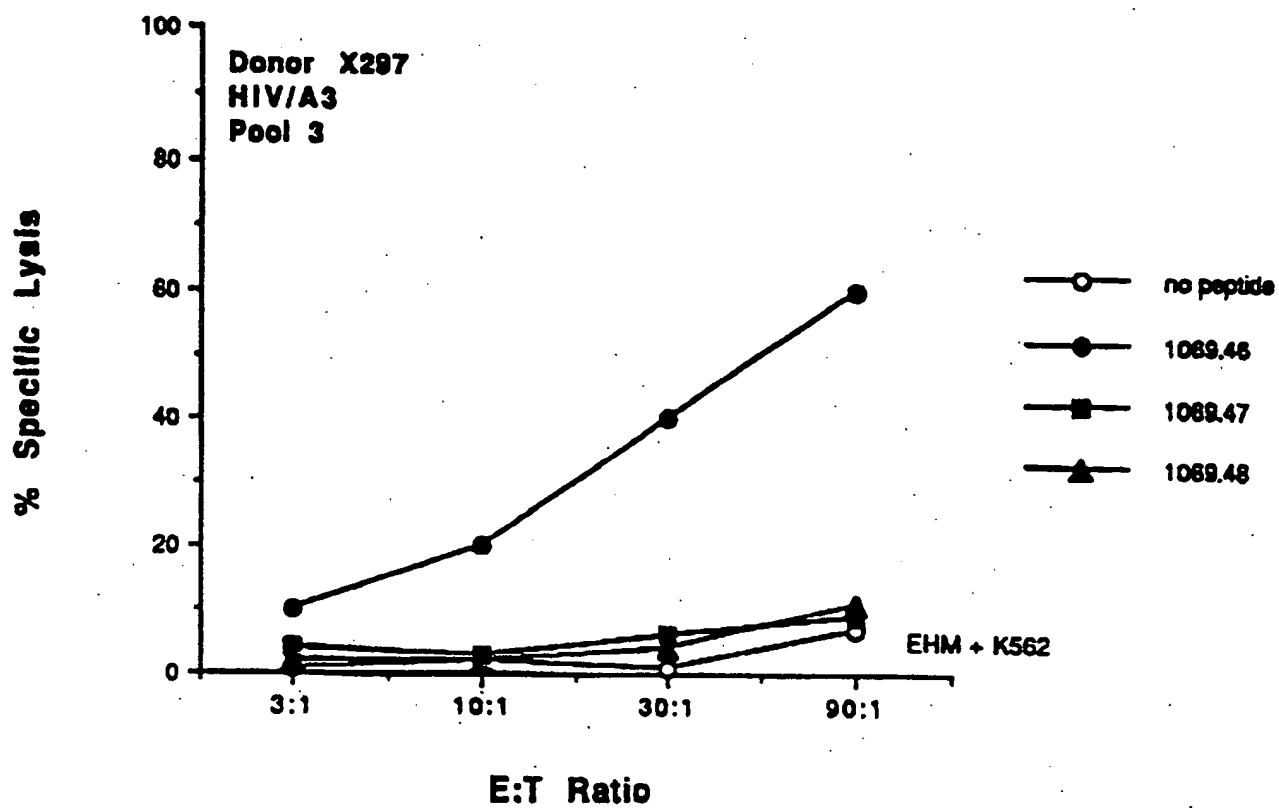
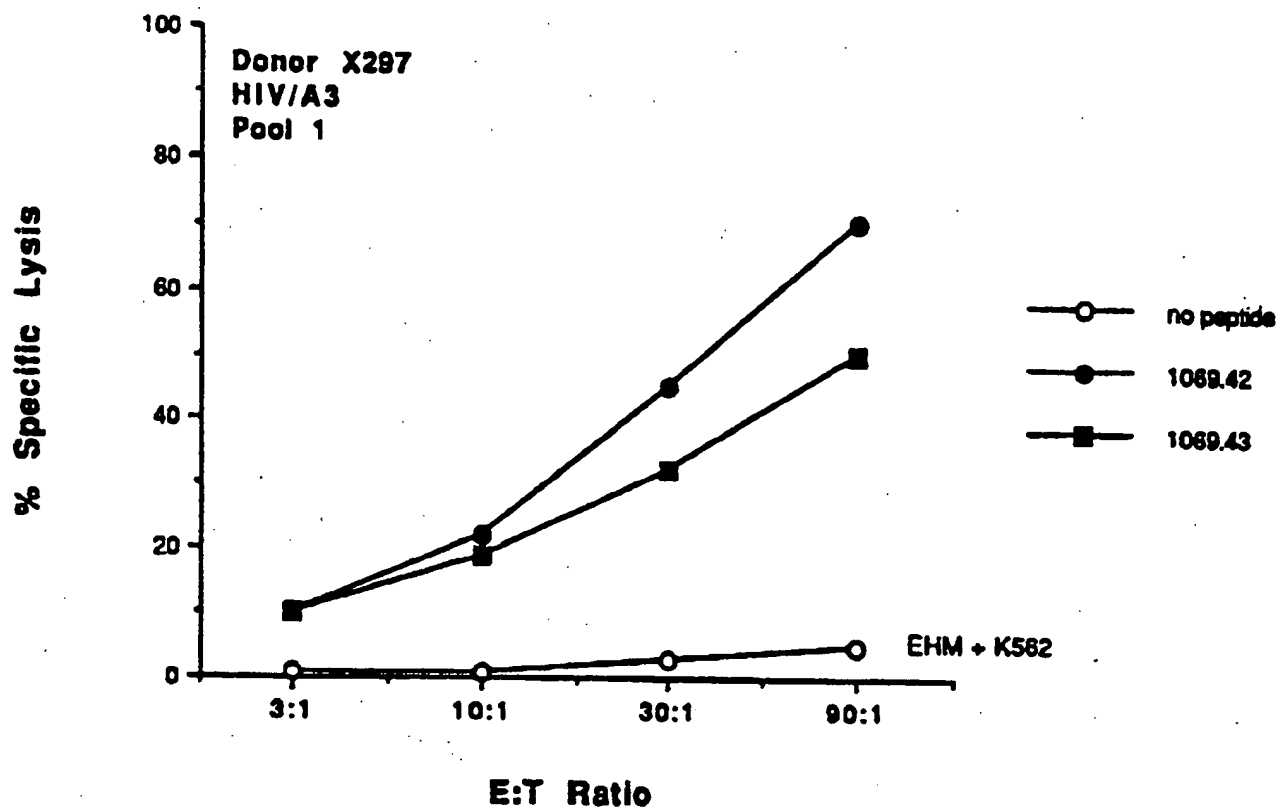
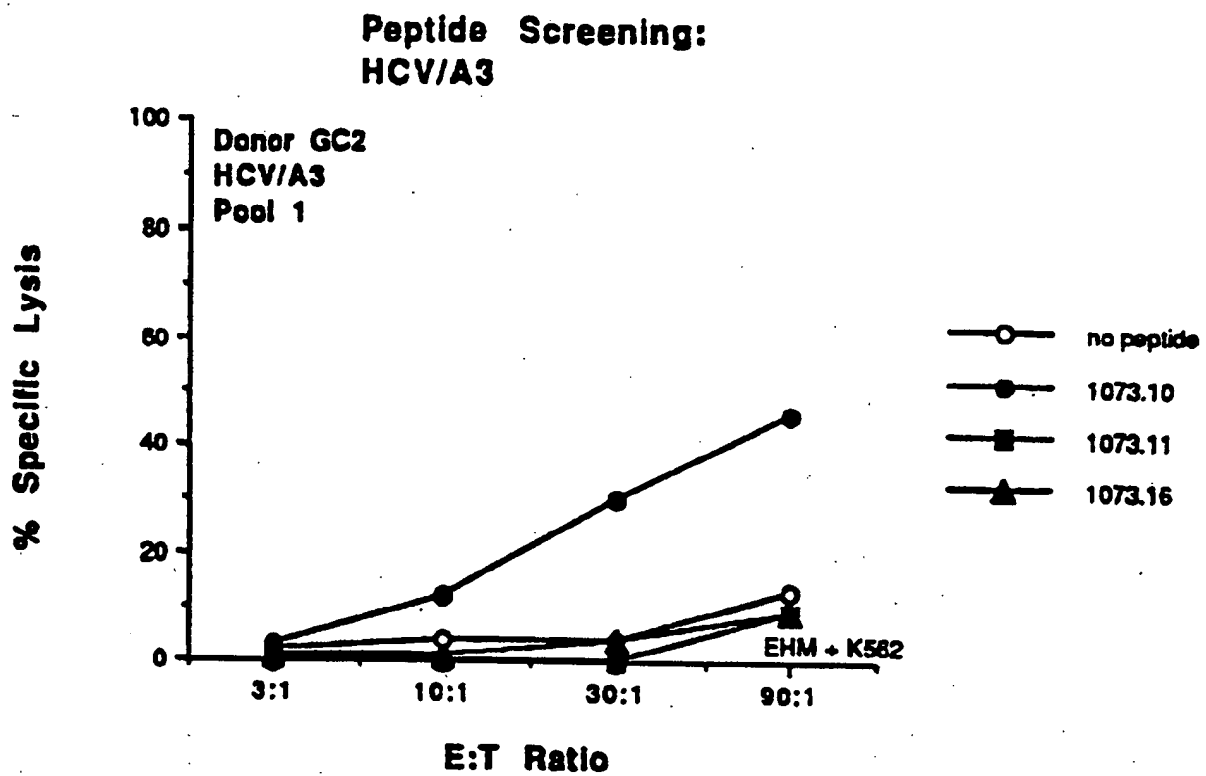
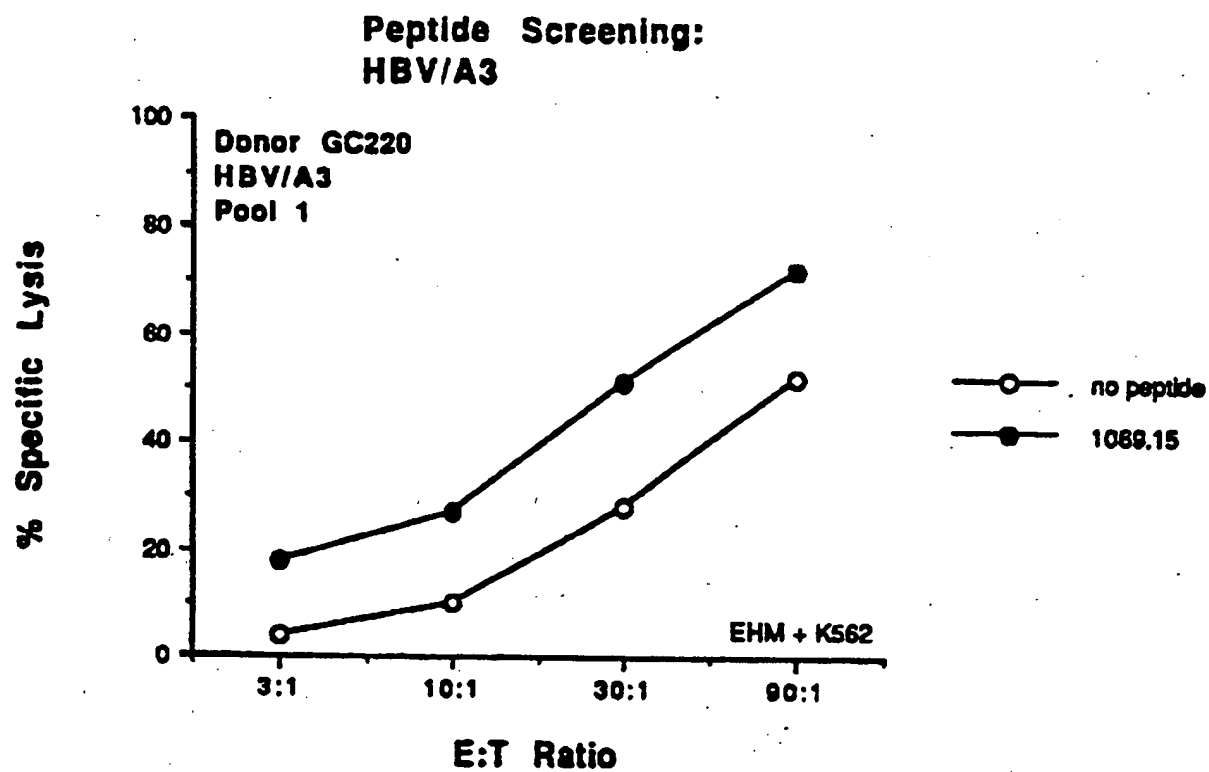
**Peptide Screening:
HIV/A3**

Figure 24



25/25

Figure 25



PROSTATE STEM CELL ANTIGEN: IDENTIFICATION OF IMMUNOGENIC PEPTIDES AND ASSESSMENT OF REACTIVE CD8⁺ T CELLS IN PROSTATE CANCER PATIENTS

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Identification of TAAs recognized by CD8⁺ CTLs paved the way for new concepts in cancer therapy. In view of the heterogeneity of tumors and their diverse escape mechanisms, CTL-based cancer therapy largely depends on an appropriate number of TAAs. In prostate cancer, the number of antigens defined as suitable targets of CTLs remains rather limited. PSCA is widely distributed in prostate cancer. In this report, we define immunogenic peptides of PSCA which are recognized by circulating CD8⁺ T cells from prostate cancer patients and able to activate CTLs *in vitro*. Screening the amino acid sequence of PSCA for peptides containing a binding motif for HLA-A*0201 resulted in 8 candidate peptides. Specificity and affinity of peptide binding were verified in a competition assay. Frequencies of CD8⁺ T lymphocytes reactive against selected epitopes were determined in the blood of prostate cancer patients using the ELISPOT assay. Increased frequencies were revealed for CD8⁺ T cells recognizing the peptides ALQPGTALL and AILALLPAL. CTLs from prostate cancer patients were raised against these 2 peptides *in vitro* when presented by autologous DCs. They specifically recognized peptide-pulsed T2 target cells and prostate cancer cells that were HLA-A*0201- and PSCA-positive, indicating that these peptides were naturally generated by tumor cells. These data suggest that PSCA is a promising target for the immunotherapy of prostate cancer.

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Key words: prostate stem cell antigen; T cell; tumor antigen; dendritic cell; immunotherapy

Prostate cancer is the most common cancer diagnosis and the second leading cause of cancer-related death in men.¹ Treatment of advanced disease is restricted to chemotherapy and hormonal therapy. Because of the frequent development of tumor escape variants resistant to both types of therapy, alternative treatment modalities are required.

In recent years, immunotherapy of human tumors has advanced with the finding that CD8⁺ CTLs are capable of recognizing and destroying tumor cells that expose peptides derived from TAAs and bound to MHC class I molecules.² In addition, clinical studies have clearly demonstrated the potential of adoptive immunotherapy based on CD8⁺ effector T cells.^{3,4} So far, HLA allele-specific peptides from a number of well-characterized prostate-associated antigens have been identified as CD8⁺ T-cell epitopes. HLA-A*0201-binding peptides derived from PSA,⁵ PSMA⁶ and PAP⁷ have been described as targets of tumor-reactive CTLs *in vitro*.

In addition, the telomerase catalytic subunit⁸ and the apoptosis inhibitor protein survivin^{9,10} are widely distributed in human tumor tissues, including prostate cancer. Peptides derived from these proteins elicit peptide-specific cytotoxic T-cell responses *in vitro*.^{11–13}

Attempts to induce systemic CD8⁺ T-cell immunity to prostate-associated antigens have been introduced into the treatment of prostate cancer. In a clinical trial, patients with advanced prostate cancer received infusions of autologous DCs pulsed with PSMA-derived peptides. Vaccination was well tolerated, and objective

clinical responses were evident in over 30% of evaluated patients.¹⁴ In another clinical study, infusion of autologous DCs pulsed with a fusion protein consisting of PAP linked to GM-CSF was followed by declining PSA levels in some patients.¹⁵

PSCA was identified by PCR-based subtractive hybridization.¹⁶ PSCA is predominantly expressed on the surface of prostate cells and overexpressed by both androgen-dependent and androgen-independent prostate tumors. It has also been found in transitional cell carcinoma of the bladder¹⁷ and pancreatic cancer.¹⁸ It belongs to a family of glycosylphosphatidylinositol-anchored glycoproteins, and its amino acid sequence shares 30% identity with stem cell antigen 2.¹⁶ *In situ* hybridization and immunohistochemical analysis revealed PSCA expression in over 80% of primary prostate carcinomas and in all bone metastases examined.^{16,19} A strong correlation between the level of PSCA protein expression and tumor stage, histologic grade and progression to androgen independence has been documented.¹⁹

Previous studies have pointed out the suitability of PSCA as a target antigen in specific immunotherapy. Firstly, anti-PSCA MAbs inhibited tumor growth and formation of metastases in mice.²⁰ Secondly, *in vitro* stimulation with an HLA-A*0201-binding, PSCA-derived peptide induced a tumor-specific CTL response.²¹

In this study, CD8⁺ T cells reactive against 2 of 8 predicted HLA-A*0201-binding PSCA peptides were detected in the blood of prostate cancer patients. In addition, these peptides efficiently activated CD8⁺ effector T cells, which are capable of destroying prostate cancer cells, indicating their autochthonous presentation.

Abbreviations: CTL, cytotoxic T cell; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; HS, human serum; MAbs, monoclonal antibody; PAP, prostate acid phosphatase; PBMC, peripheral blood mononuclear cell; PBST, PBS + 0.05% Tween; PSA, prostate-specific antigen; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; TAA, tumor-associated antigen.

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MATERIAL AND METHODS

Cell lines

The prostate cancer cell line PC-3 as well as the HLA-A*0201-positive mutant cell line T2 and the chronic myelogenous leukemia cell line K-562 (ATCC, Manassas, VA) were cultured according to the provider's instructions. The prostate cancer cell line LNCaP 1740 (ATCC) was cultured in RPMI-1640 medium with 25 mM HEPES buffer and L-glutamine (Life Technologies, Karlsruhe, Germany) supplemented with 1% nonessential amino acids (Biochrom, Berlin, Germany) and 10% heat-inactivated FCS (Biochrom). The melanoma cell line 93.04A12.1 was kindly provided by Dr. C.J.M. Melief (University Hospital, Leiden, the Netherlands). This cell line was maintained in RPMI-1640 medium (Biochrom) supplemented with 2 mM L-glutamine, 10 mM sodium pyruvate and 1% nonessential amino acids (all from Biochrom), 100 µg/ml penicillin, 100 µg/ml streptomycin (both from Life Technologies) and 10% heat-inactivated FCS.

MHC class I type of cell lines LNCaP 1740, PC-3 and 93.04A12.1 was determined by PCR analysis at the HLA-typing laboratory of the Institute of Immunology (Technical University of Dresden): LNCaP 1740: HLA-A*01, *0201, HLA-B*08, *37, HLA-Cw*06, *07; PC-3: HLA-A*01, *24, HLA-B*13, *55, HLA-Cw*01, *06; 93.04A12.1: HLA-A*0201, *24, HLA-B*40, *44, HLA-Cw*03, *05.

Expression of HLA class I molecules on the cell surface of LNCaP 1740, PC-3 and 93.04A12.1 cells stimulated with 200 U/ml IFN-γ for 48 hr was analyzed by cytofluorometry using a FITC-conjugated mouse antihuman HLA-A, -B, -C MAb (clone G46-2.6; Pharmingen, San Diego, CA). Mean fluorescence intensity was 170, 705 and 2,429 arbitrary units for cell lines LNCaP 1740, PC-3 and 93.04A12.1, respectively. Expression of HLA-A2 molecules was determined by incubation with the anti-HLA-A2 MAb MA2.1 (ATCC) or with mouse IgG₁ (Pharmingen) as an isotype control, followed by staining with an antimouse IgG Fc-directed F(ab')₂ fragment coupled to phycoerythrin (Immunotech, Marseille, France). Whereas the 93.04A12.1 cell line was strongly stained by the MA2.1 MAb, LNCaP 1740 expressed HLA-A2 only at low density, yet it was regularly detected on >85% of cells.

Blood and tumor samples

All blood and tumor samples were obtained from prostate cancer patients and healthy donors with informed consent. Blood and tissue specimens were obtained from prostatectomized patients together with clinical information and pathological reports. Preoperative serum levels of PSA were 0.4–38.4 ng/ml.

Detection of PSCA transcripts in prostate tissue by RT-PCR

Prostate tissue specimens obtained during prostatectomy and immediately stored in liquid nitrogen were available from 6 of 11 prostate carcinoma patients. Total RNA was extracted by standard procedures (Trizol LS Reagent; Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Purified RNA diluted with sterile water was quantitated and assessed for purity by UV spectrophotometry. After a DNA digestion step, cDNA synthesis was performed using random hexamer primers (Ready to Go You Prime First Strand Kit; Amersham, Freiburg, Germany). RNA quality was tested using a standard glyceraldehyde-3-phosphate dehydrogenase amplification protocol. The primer pair 5'-ATG-AAGGCTGTGCTGCTTGCC-3'/5'-CTATAGCTGGCCGGT-CCCCA-3' was used to assess PSCA mRNA expression with a touch-down cycling profile of 94°C for 30 sec, 65°C for 30 sec and 68°C for 1 min (5 cycles), followed by 94°C for 30 sec, 62°C for 30 sec and 68°C for 1 min (35 cycles) and 1 additional round at 68°C for 5 min. As positive controls, we used cDNA from LNCaP 1740 and PC-3 cells.²¹

Preparation of DCs

Blood DCs were isolated using the MAb M-DC8, as described previously.²² Briefly, PBMCs were prepared from blood samples by Ficoll-Hypaque (Biochrom) density centrifugation. M-DC8⁺

DCs were obtained by incubating PBMCs for 15 min at 4°C with diluted supernatant of the M-DC8 hybridoma containing 10 µg/ml of antibody. After washing with PBS, 1×10^8 cells were resuspended in 500 µl of PBS and labeled with 10 µl of rat antimouse IgM coupled to paramagnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for another 15 min at 4°C. After washing, cells were thoroughly resuspended to avoid aggregation and sorted on a VS+ separation column (Miltenyi Biotec). If necessary, the highly enriched cells were further purified by a second round of magnetic cell sorting. Degassed, ice-cold PBS containing 1% HS (CC pro, Neustadt, Germany) was used as running buffer and for elution after removal of the magnetic field. By this technique, M-DC8⁺ DCs with a purity of >95% were prepared from PBMCs that contained 0.5–2% of this DC population.

Monocytes were isolated by immunomagnetic cell separation with anti-CD14 antibody coupled to paramagnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions. To generate immature DCs, monocytes were cultured in the presence of 1,000 U/ml GM-CSF and 1,000 U/ml IL-4 (both from Strathmann Biotec, Hanover, Germany) in RPMI-1640 medium supplemented with 10% HS for 7 days. For further maturation, DCs were cultured for an additional 3 days in the presence of 1,000 U/ml GM-CSF, 1,000 U/ml IL-4, 1,000 U/ml IL-6, 10 ng/ml tumor necrosis factor-α, 10 ng/ml IL-1β (all from Strathmann Biotec) and 1 µg/ml prostaglandin E₂ (Sigma-Aldrich, Steinheim, Germany) in RPMI-1640 medium supplemented with 10% HS.

Epitope prediction

Epitope prediction was done as described.²³ Briefly, potential HLA-A*0201 ligands were selected from the sequence of PSCA (accession AF043498) using a matrix pattern suitable for the calculation of nonamer or decamer peptides fitting the HLA-A*0201 motif. Such motif-based epitope predictions are available on the web (<http://www.syfpeithi.de>).

Peptides

Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/Bu strategy. After removal from the resin by treatment with trifluoroacetic acid/phenol/ethanedithiol/thioanisole/water (90/3.75/1.25/2.5/2.5 by vol) for 1 or 3 hr in the case of arginine-containing peptides, synthesis products were precipitated from methyl-tert butyl ether, washed once with methyl-tert butyl ether and twice with diethyl ether and resuspended in water prior to lyophilization. Synthesis products were analyzed by HPLC (Varian Star; Zinsser, Munich, Germany) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (G2025A; Hewlett-Packard, Waldbronn, Germany). Peptides of <80% purity were purified by preparative HPLC.

Competition assay

To identify potential HLA-A*0201-binding peptides from the amino acid sequence of PSCA, binding studies were carried out using whole cells (LCL721 or JY) and a fluorescence-based competition assay, essentially as described by Van der Burg *et al.*²⁴ but without performing acid strip. The reporter peptide was ILK(FITC)EPVHGV from HIV-1 reverse transcriptase; the positive control was YLLPAIVHI from RNA helicase p72. All binding data are expressed as percentage compared to binding of the positive control. Fluorescence intensities were recorded in a flow cytometer (FAC-SCalibur; Becton Dickinson, Heidelberg, Germany).

ELISPOT assay

Wells of MultiScreen-HA plates (Millipore, Bedford, MA) were coated with 100 µl of mouse-antihuman IFN-γ antibody (10 µg/ml, clone 1-D1K; Mabtech, Nacka, Sweden) in PBS. After incubation overnight at 4°C, unbound antibody was removed by 6 washings with PBS. Coated wells were blocked with 100 µl RPMI-1640 supplemented with 10% HS. After 1 hr at 37°C, the blocking medium was discarded. Monocytes (5×10^4) purified by

immunomagnetic separation (Miltenyi Biotec) and used as stimulator cells were plated in each well. Antigen-presenting cells were pulsed with peptides in a final volume of 100 μ l/well at a concentration of 100 μ g/ml, as previously described.^{25,26} After 2 hr incubation at 37°C, 2×10^5 CD8⁺ T cells isolated by immunomagnetic purification (Miltenyi Biotec) were added to each well. Cells were spun down at 150g for 3 min, and the medium was pressed through the mixed cellulose ester bottom of the plate. After addition of 100 μ l fresh RPMI-1640 supplemented with 10% HS per well, cells were incubated for 36 hr at 37°C. Thereafter, cells were removed by 6 washings with PBST and 100 μ l of biotinylated detection antibody against human IFN- γ (2 μ g/ml, clone 7-B6-1; Mabtech) were added per well. After 2 hr at 37°C, plates were rinsed 6 times with PBST. Then, 100 μ l of an avidin-biotin peroxidase complex preparation (ABC Vectastain-Elite kit; Vector, Burlingame, CA) were added to each well at a dilution of 1:100 and incubated for 1 hr at room temperature. Unbound complexes were removed by 3 washings with PBST and 3 washings with PBS. The staining reaction was started by adding a solution of 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO) dissolved according to the manufacturer's instructions. The reaction was stopped after 4 min by washing the plates under running tap water. Spots were counted using a stereomicroscope (Zeiss, Jena, Germany) at $\times 40$ magnification. The cut-off for positive spots was defined as a spot size greater than $3 \times$ SD above the mean value of the spot diameter obtained in the presence of the HIV control peptide.

In vitro generation of PSCA-specific CTLs

To generate PSCA-specific CTLs, freshly isolated M-DC8⁺ DCs were pulsed with the individual PSCA-derived peptides at a concentration of 20 μ g/ml in serum-free RPMI-1640 medium for 2–4 hr at 37°C. This peptide concentration has been suitable for activation of T cells against various peptides in a series of experiments. After washing, 2×10^5 peptide-loaded M-DC8⁺ DCs were cocultured with 2×10^6 CD8⁺ T cells isolated by immunomagnetic separation in 2 ml RPMI-1640 medium supplemented with 10% HS per well of a 24-well tissue culture plate (Greiner, Frickenhausen, Germany). Seven days later, cultures were washed and restimulated with peptide-loaded M-DC8⁺ DCs or, if necessary, with monocyte-derived DCs at a responder-to-stimulator ratio of 5:1 and supplemented with 100 U/ml IL-2 and 10 ng/ml IL-7 (both from Strathmann Biotech). After 3–5 cycles of stimulation, cultures were tested for the presence of PSCA-specific CTLs.

Cr-release assay

The cytotoxic activity of *in vitro* stimulated CTLs was tested against the HLA-A*0201-positive mutant cell line T2, different prostate cancer cell lines, the melanoma cell line 93.04A12.1 and the chronic myelogenous leukemia cell line K-562 as targets in a 4 hr standard ⁵¹Cr-release assay. Briefly, tumor cell lines were labeled for 1 hr at 37°C with 100 μ Ci ⁵¹Cr (sodium chromate; NEN, Zaventem, Belgium) in 1 ml RPMI-1640 medium. To assay

the cytotoxic activity of cultured T cells against the predicted PSCA peptides, T2 cells were incubated for 4 hr with individual peptides at a concentration of 50 μ g/ml and then labeled with ⁵¹Cr and used as target. The peptide concentration used for loading of T2 cells corresponded to that used by other authors.^{27,28} Cr-labeled target cells were washed 3 times and plated in round-bottomed 96-well plates at 5×10^3 cells/well. Effector cells were added as triplicates at different ratios. For cold target inhibition experiments, a 20-fold excess of cold T2 cells loaded with one of the PSCA peptides or an irrelevant peptide derived from HIV-1 reverse transcriptase was added to the ⁵¹Cr-labeled LNCaP 1740 cells.

After 4 hr incubation, 100 μ l of supernatant were collected from each well and the released ⁵¹Cr was determined in a β -counter (Wallac, Freiburg, Germany). Maximal release and spontaneous release were measured by treating labeled cells with 2% Triton X-100 (Ferah, Berlin, Germany) or medium alone, respectively. Specific cytotoxicity was calculated according to the following formula:

$$\text{Percent specific lysis} = 100$$

$$\times [(\text{cpm experimental release}$$

$$- \text{cpm spontaneous release}) / (\text{cpm maximal release}$$

$$- \text{cpm spontaneous release})]$$

RESULTS

Selection of PSCA peptides binding to HLA-A*0201

The amino acid sequence of PSCA was screened for peptides containing the HLA-A*0201 peptide motif. Eight peptides were selected according to their score (Table I). Peptides were examined for binding to HLA-A*0201 by a competition assay. Peptides ALQPGTALL (0374), AILALLPAL (0376), LLLWGPQQL (0378), ALLMAGLAL (0380), LLALLMAGL (0347) and ALLPALGLL (0354) bound with high affinity, whereas peptides ALQPAAAIL (0375) and RIRAVGLLTV (0355) bound with intermediate affinity (Table I). All peptides were used to detect reactive T cells in the blood of prostate cancer patients.

Detection of PSCA peptide-reactive CD8⁺ T cells in the blood of prostate cancer patients

By ELISPOT analysis, blood samples of 8 prostate cancer patients were tested for the presence of PSCA peptide-reactive CD8⁺ T cells. Clinical data of all patients are summarized in Table II. Expression of PSCA in resected tissue was analyzed by RT-PCR. PSCA transcripts could be detected in all specimens available (Fig. 1, lanes 1–6; Table II).

To determine the frequency of PSCA peptide-reactive CD8⁺ T cells by the IFN- γ ELISPOT assay, monocytes were prepared by immunomagnetic separation from the blood of 8 prostate cancer patients expressing the HLA-A*0201 allele. Monocytes were loaded with PSCA-derived peptides and then cocultured with

TABLE I—PSCA PEPTIDES SELECTED BY EPITOPE PREDICTION AND TESTED FOR BINDING TO HLA-A*0201 BY COMPETITION ASSAY

Peptide	Position ¹	Sequence	m.w.	Length ²	Score	BA (%) ³
0347	5–13	LLALLMAGL	913.6	9	28	91.9
0354	108–116	ALLPALGLL	879.6	9	30	102.7
0355	52–61	RIRAVGLLTV	1096.7	10	25	68.6
0374	14–22	ALQPGTALL	882.5	9	30	92.7
0375	99–107	ALQPAAAIL	866.5	9	26	67.9
0376	105–113	AILALLPAL	893.6	9	29	108.6
0378	115–123	LLLWGPQQL	995.6	9	26	101.7
0380	7–15	ALLMAGLAL	871.5	9	26	93.6

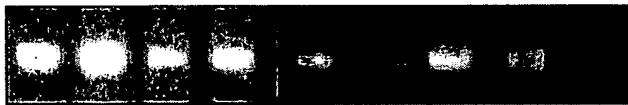
¹Values indicate position of peptide in the amino acid sequence of PSCA. ²Number of amino acids. ³Binding data are expressed as percentage in comparison to the binding of the positive control peptide YLLPAIVHI from RNA helicase p72. Reporter peptide was ILK(FITC)EPVHGV from HIV-1 reverse transcriptase. Test peptides, positive control and reporter peptide were used at a concentration of 10 μ M. BA, binding affinity.

autologous purified CD8⁺ T cells. As a positive control, monocytes were loaded with influenza matrix peptide; as a negative control, stimulator cells were either pulsed with an HIV-derived peptide or left unpulsed. Results of the ELISPOT analysis are summarized in Table III. CD8⁺ T cells reactive against influenza matrix peptide were detected in all patients. The frequency of CD8⁺ T cells reactive against peptide 0376 was increased in 4 of 8 patients. In addition, enhanced frequencies of CD8⁺ T cells reactive against peptide 0374 were detected in 2 patients. In contrast, no or only weak T-cell responses against the other 6 PSCA peptides were observed in prostate cancer patients. When, for comparison, CD8⁺ T cells purified from the blood of 3 healthy HLA-A*0201-positive donors were tested by ELISPOT assay, no response against any of the selected PSCA peptides was detected, whereas influenza-reactive CD8⁺ T cells were present in all 3 donors (Table III).

TABLE II - HISTOPATHOLOGICAL DATA AND PSCA EXPRESSION IN TUMOR SPECIMENS OF ALL ANALYZED PATIENTS

Patient	Age ¹ (years)	Staging	Grading	GS	PSCA ²
1	72	pT2NxcM0	Ib	7	+
2	69	pT2apN0cM0	IIa	8	+
3	68	pT2apN0cM0	Ib	6	+
4	71	pT4pNxcM0	Ib	8	+
5	61	pT2bpNxcM0	Ib	7	+
6	60	pT2bpN0cM0	Ib	6	+
7	68	pT2bpN0cM0	IIa	7	n.d.
8	67	pT2bpN0cM0	IIa	5	n.d.
9	60	pT2bpN0cM0	IIa	6	n.d.
10	67	pT2bpN0cM0	IIa	5	n.d.
11	68	pT2bpN0cM0	IIa	8	n.d.

¹Patient age at the time of radical prostatectomy. ²PSCA mRNA expression determined by RT-PCR in tissue specimens obtained during tumor resection. GS, Gleason score; n.d., not determined.



1 2 3 4 5 6 7 8 9

FIGURE 1 - RT-PCR analysis of PSCA mRNA expression in cell lines and tumor samples of patients included in our study. Lanes 1-6 represent PCR results of tumor tissue analysis of patients 1-6, respectively. Prostate carcinoma cell lines LNCaP 1740 (lane 7) and PC-3 (lane 8) and melanoma cell line 93.04A12.1 (lane 9).

In vitro generation of PSCA peptide-reactive CD8⁺ cytotoxic effector T cells

To evaluate the capacity of the 2 identified PSCA peptides to activate cytotoxic effector T cells *in vitro*, CD8⁺ T lymphocytes were purified from PBMCs of 3 prostate cancer patients (patients 9-11). Clinical data of these patients are shown in Table II. CD8⁺ T cells were subjected to 3-5 weekly stimulations with autologous peptide-loaded DCs. Specific cytotoxicity was determined in a Cr-release assay with peptide-pulsed T2 cells as targets. When CD8⁺ T lymphocytes from prostate cancer patients 9 and 10 were stimulated with peptide 0376, efficient lysis of T2 target cells loaded with this peptide was observed whereas only background lysis was detected with unloaded T2 cells (Fig. 2a). To determine whether peptide 0376 originates from natural processing of PSCA and is presented on the surface of tumor cells, peptide-stimulated CD8⁺ T cells of the 2 donors were tested against the HLA-A*0201-positive and PSCA-positive prostate cancer cell line LNCaP 1740, the HLA-A*0201-negative and PSCA-positive prostate cancer cell line PC-3 as well as the HLA-A*0201-positive and PSCA-negative melanoma cell line 93.04A12.1. PSCA expression of these cell lines was determined by qualitative RT-PCR (Fig. 1, lanes 7-9). To exclude natural killer cell-like activity, K-562 cells were also used as targets. To increase the processing and presentation of endogenous antigens, tumor cells were cultured in medium supplemented with 200 U/ml IFN-γ over 48 hr before being used as target cells in the Cr-release assay. Peptide-stimulated T cells from all donors efficiently lysed LNCaP 1740 cells, whereas only weak lysis was observed against PC-3, 93.04A12.1 and K-562 cells (Fig. 2b,c). Peptide specificity of CTL-mediated killing was further demonstrated by cold target inhibition experiments. As shown in Figure 2d, lysis of LNCaP 1740 cells was specifically blocked by cold T2 target cells pulsed with peptide 0376, whereas no significant inhibition of lysis was observed when unlabeled T2 cells were pulsed with the irrelevant HIV peptide.

Stimulation of CD8⁺ T lymphocytes from prostate cancer patients 9 and 11 with peptide 0374 resulted in the lysis of T2 cells loaded with this peptide (Fig. 3a). These CD8⁺ T cells exhibited marked lysis of LNCaP 1740 cells yet were unable to lyse PC-3, 93.04A12.1 and K-562 cells to a significant degree (Fig. 3b,c). Specificity of lysis was again verified by cold target inhibition (Fig. 3d).

DISCUSSION

Expression of the recently identified protein PSCA is restricted to prostate tissue, prostate carcinoma cells and some other tumors. Remarkably, it is overexpressed in both androgen-dependent and androgen-independent prostate tumors, including bone metastases, and appears to be upregulated in advanced tumors.¹⁹ In the present

TABLE III - DETECTION OF CD8⁺ T-CELL REACTIVITY AGAINST HLA-A*0201-BINDING PSCA PEPTIDES IN THE BLOOD OF PROSTATE CANCER PATIENTS BY ELISPOT ASSAY

Peptide	Patient								Healthy Donor		
	1	2	3	4	5	6	7	8	1	2	3
No peptide	0	0	0	0	0	0	0	0	0	0	0
Influenza	47.0	20.0	16.0	15.7	102.3	32.7	230.0	10.3	14.0	8.3	11.3
PSCA 0347	0	0	0.7	0	n.d.	n.d.	0.7	0	n.d.	n.d.	n.d.
PSCA 0354	2.7	0	0.7	0	1.3	n.d.	0	0.7	0	0	0
PSCA 0355	0.7	0.7	0	0	1.0	n.d.	0	0	n.d.	n.d.	n.d.
PSCA 0374	3.7	0	0	0	57.3	27.0	0	4.7	0	0	0
PSCA 0375	0	0.7	0	0	1.0	n.d.	0	0	n.d.	n.d.	n.d.
PSCA 0376	36.0	15.3	0	0	50.3	30.3	0.7	1.0	0.7	0	0
PSCA 0378	3.7	2.3	0	0.7	2.3	n.d.	0	1.3	0	0	0
PSCA 0380	2.3	2.3	0.7	1.3	0.7	n.d.	0	0	0.7	0	0.7

Monocytes of 8 prostate cancer patients and 3 healthy donors were pulsed with 100 μg/ml of HIV peptide ILKEPVHGV from reverse transcriptase, influenza peptide GILGFVFTL from matrix protein M1 or one of the PSCA peptides or left unpulsed; Monocytes were then used as antigen-presenting cells. Monocytes (5×10^4) were cocultured for 36 hr with 2×10^5 purified CD8⁺ T cells. Results are presented as means of triplicate determinations. SEM was <15% for all determinations. Relevant reactivities are marked in bold. n.d., not determined.

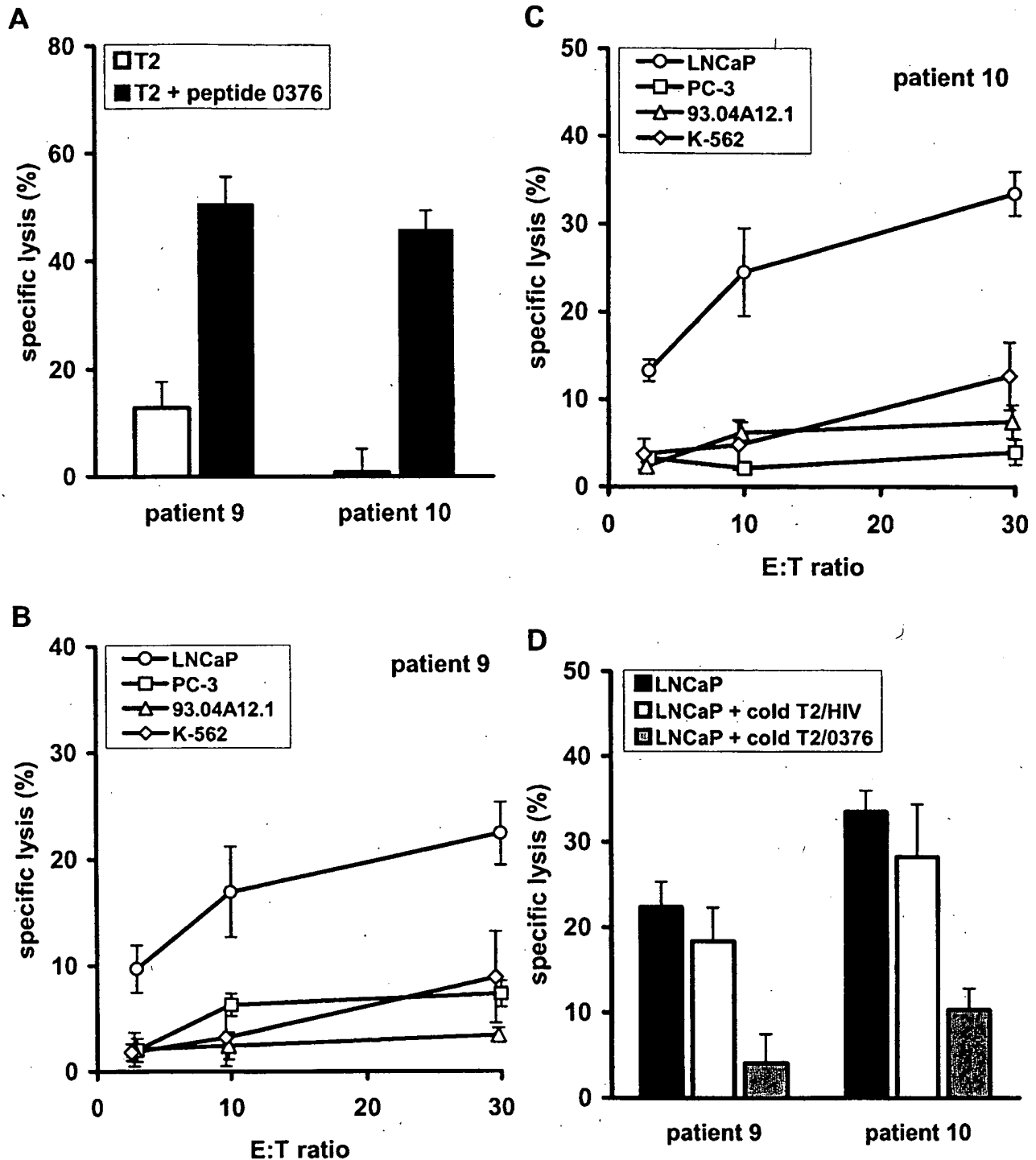


FIGURE 2 – Recognition of endogenously produced PSCA-derived peptide 0376 by PSCA-reactive CTLs. Purified CD8⁺ T lymphocytes from the blood of 2 prostate cancer patients were activated by consecutive rounds of stimulation against PSCA-derived nonamer peptide 0376 presented by purified autologous DCs. (a) Peptide-loaded, HLA-A*0201-positive T2 cells were labeled with ⁵¹Cr. The different T-cell cultures were added at an E:T ratio of 20:1. T2 cells without PSCA-derived peptide served as controls. After 4 hr of incubation, Cr release was measured. (b,c) Activated CD8⁺ T cells were cocultured with 5×10^3 ⁵¹Cr-labeled LNCaP 1740, PC-3, 93.04A12.1 or K-562 tumor cells/well at various E:T ratios (3:1, 10:1, 30:1). After 4 hr-incubation, Cr release was measured. (d) Cold target inhibition of LNCaP 1740 cell lysis was performed at an E:T ratio of 30:1. Cold T2 cells pulsed with HIV peptide ILKEPVHGV from reverse transcriptase or PSCA-derived peptide 0376 were added at a cold:hot ratio of 20:1. All results are presented as means \pm SEM of triplicate determinations.

study, we show by ELISPOT analysis that PSCA-derived peptides are recognized by CD8⁺ T cells in the blood of prostate cancer patients.

The ELISPOT assay has become a favored and widely employed method for quantification of T lymphocytes reactive against TAA-derived epitopes. For instance, marked ELISPOT

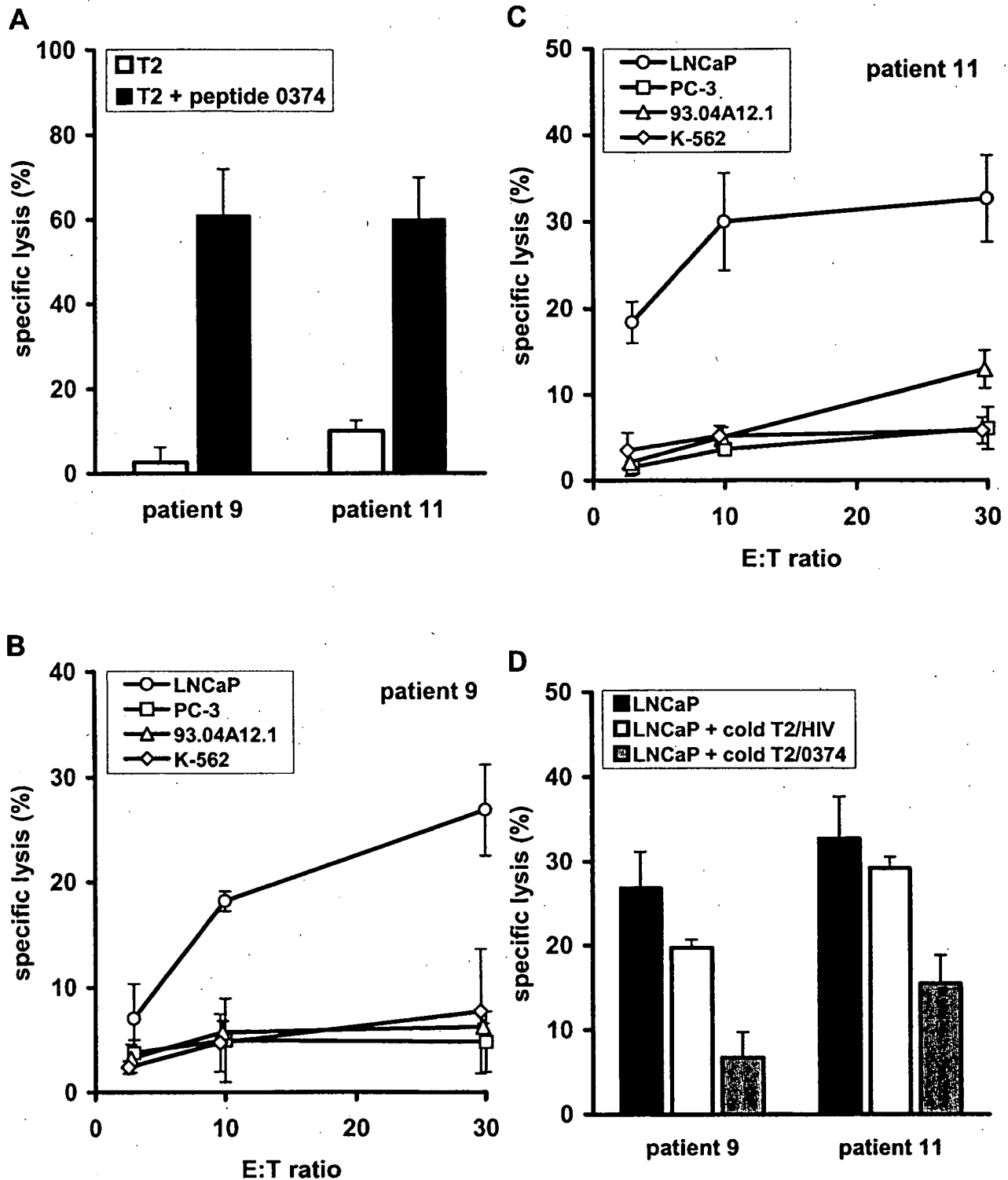


FIGURE 3—Generation of cytotoxic effector cells specific for PSCA-derived peptide 0374. Purified CD8⁺ T lymphocytes from 2 prostate cancer patients were stimulated weekly with purified autologous DCs loaded with PSCA-derived nonamer peptide 0374. (a) Peptide-pulsed, HLA-A*0201-positive T2 cells were labeled with ⁵¹Cr. The different T-cell cultures were added at an E:T ratio of 20:1. T2 cells without PSCA-derived peptide served as controls. After 4 hr of incubation, Cr release was measured. (b,c) Activated CD8⁺ T cells were cocultured with ⁵¹Cr-labeled 5 × 10³ LNCaP 1740, PC-3, 93.04A12.1 or K-562 cells/well at various E:T ratios (3:1, 10:1, 30:1). After 4 hr of incubation, Cr release was measured. (d) Peptide-specific blocking of LNCaP 1740 cell lysis was analyzed by cold target inhibition at an E:T ratio of 30:1. Unlabeled T2 cells loaded with HIV peptide ILKEPVHGV from reverse transcriptase or PSCA-derived peptide 0374 were added at a cold:hot ratio of 20:1. All results are presented as means ± SEM of triplicate determinations.

reactivity against peptides of the apoptosis inhibitor protein survivin, which had previously been identified by inducing CTL responses,¹³ were detected in the blood of tumor patients.²⁹ In another study, strong CD8⁺ T-cell reactivity against NY-ESO-1-derived peptides was found by ELISPOT assay in patients with stage IV melanoma and other types of advanced cancer.³⁰ In addition, ELISPOT analysis allowed detection of NY-ESO-1-reactive CD4⁺ T cells in the blood of melanoma patients. In this approach, the HLA class II-binding peptides were defined by T-cell proliferation assays.³¹

Taking into account that HLA-A*0201 is the most frequent HLA-A allele in Caucasian individuals, we used epitope prediction to select 8 PSCA-derived peptides that bound to HLA-A*0201 molecules as determined by a competition assay. Peptides that bind HLA-A*0201 with high affinity are predominantly nonamers and decamers with allele-specific anchor residues at positions 2 and 9 for nonamers and positions 2 and 10 for decamers.²³ Typical anchor residues are isoleucine (I) or leucine (L) at position 2 and valine (V), leucine (L) or methionine (M) at the C-terminal position. All selected PSCA peptides fulfilled the requirements for HLA-A*0201 binding.

Of this collection, peptides 0376 and 0374 were recognized by an increased number of circulating CD8⁺ T cells from prostate cancer patients when analyzed by ELISPOT assay. Tumor peptide-reactive CD8⁺ T cells have been mainly observed in patients with advanced tumors,^{30,32,33} whereas our data provide evidence that such T lymphocytes can also be found in patients with localized, nonmetastatic disease. Detection of PSCA peptide-reactive CD8⁺ T-cell responses in patients who also express PSCA in their tumors suggests that PSCA can serve as an immunogenic TAA *in vivo*. This observation is in accordance with the previous description of CD8⁺ T-cell responses against NY-ESO-1-derived peptides occurring only in patients with NY-ESO-1-expressing tumors.³⁰

PSCA peptides 0376 and 0374 were further substantiated as candidate targets for the immunotherapy of prostate cancer by evaluating their ability to induce CTL responses *in vitro* when presented by DCs for stimulation. The specificity of lysis was tested employing peptide-loaded T2 cells. Peptide-specific CTLs efficiently lysed peptide-loaded T2 cells. Moreover, these CTL populations lysed the HLA-A*0201-positive, PSCA-expressing prostate cancer line LNCaP 1740. Lysis of LNCaP 1740 cells by PSCA peptide-activated CD8⁺ T cells was peptide-specific and HLA-restricted, as shown by a number of control experiments: killing of LNCaP 1740 cells was inhibited by adding unlabeled T2 cells pulsed with PSCA peptides 0376 or 0374, whereas no significant blockade of lysis was observed when cold T2 cells were pulsed with an irrelevant peptide. Furthermore, neither the HLA-A*0201-negative and PSCA-positive prostate cancer cell line PC-3 nor the HLA-A*0201-positive and PSCA-negative melanoma cell line 93.04A12.1 was lysed. In addition, natural killer cell-like activity was excluded by the absence of significant lysis of K-562 cells. These data confirm the endogenous origin of both peptides and their presentation by LNCaP 1740 cells.

Stimulated CTLs from a prostate cancer patient have been described to recognize peptide PSCA₁₄₋₂₂, which corresponds to peptide 0374.²¹

In summary, we show circulating CD8⁺ T cells reactive against 2 HLA-A*0201-binding PSCA-derived peptides in prostate cancer patients and specific lysis of tumor cells by CTLs activated against these peptides. Our results emphasize the suitability of PSCA as a target molecule for the immunotherapy of prostate cancer.

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REFERENCES

- Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1998. *CA Cancer J Clin* 1998;48:6-29.
- Rosenberg SA. Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol Today* 1997;18:175-82.
- Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, Simpson C, Carter C, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med* 1988;319:1676-80.
- Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 1994;86:1159-66.
- Correale P, Walmsley K, Zaremba S, Zhu M, Schlom J, Tsang KY. Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligopeptide peptide. *J Immunol* 1998;161:3186-94.
- Tjoa B, Boynton A, Kenny G, Ragde H, Misrock SL, Murphy G. Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* 1996;28:65-9.
- Peshwa MV, Shi JD, Ruegg C, Laus R, van Schooten WC. Induction of prostate tumor-specific CD8⁺ cytotoxic T-lymphocytes *in vitro* using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. *Prostate* 1998;36:129-38.
- Sommerfeld HJ, Meeker AK, Piatyszek MA, Bova GS, Shay JW, Coffey DS. Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res* 1996;56:218-22.
- Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. *Nat Med* 1997;3:917-21.
- Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, Lal A, Wang CJ, Beaudry GA, Ciriello KM, Cook BP, Dufault MR, et al. Analysis of human transcriptomes. *Nat Genet* 1999;23:387-8.
- Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. *Immunity* 1999;10:673-9.
- Minerv B, Hipp J, Firat H, Schmidt JD, Langlade-Demoyen P, Zanetti M. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc Natl Acad Sci USA* 2000;97:4796-801.
- Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, Rammensee H-G, Rieber EP. Generation of survivin-specific CD8⁺ T effector cells by dendritic cells pulsed with protein or selected peptides. *Cancer Res* 2000;60:4845-9.
- Murphy GP, Tjoa BA, Simmons SJ, Jarisch J, Bowes VA, Ragde H, Rogers M, Elgamal A, Kenny GM, Cobb OE, Ireton RC, Troychak MJ, et al. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. *Prostate* 1999;38:73-8.
- Small EJ, Fratesi P, Reese DM, Strang G, Laus R, Peshwa MV, Valone FH. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. *J Clin Oncol* 2000;18:3894-903.
- Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON. Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci USA* 1998;95:1735-40.
- Amara N, Palapattu GS, Schrage M, Gu Z, Thomas GV, Dorey F, Said J, Reiter RE. Prostate stem cell antigen is overexpressed in human transitional cell carcinoma. *Cancer Res* 2001;61:4660-5.
- Argani P, Rosty C, Reiter RE, Wilentz RE, Murugesan SR, Leach SD, Ryu B, Skinner HG, Goggins M, Jaffee EM, Yeo CJ, Cameron JL, et al. Discovery of new markers of cancer through serial analysis of gene expression. *Cancer Res* 2001;61:4320-4.
- Gu Z, Thomas G, Yamashiro J, Shintaku IP, Dorey F, Raitano A, Witte ON, Said JW, Loda M, Reiter RE. Prostate stem cell antigen (PSCA) expression increases with high Gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* 2000;19:1288-96.
- Saffran DC, Raitano AB, Hubert RS, Witte ON, Reiter RE, Jakobovits A. Anti-PSCA MAbs inhibit tumor growth and metastasis formation and prolong the survival of mice bearing human prostate cancer xenografts. *Proc Natl Acad Sci USA* 2001;98:2658-63.
- Dannull J, Diener P-A, Prikler L, Fürstenberger G, Cerny T, Schmid U, Ackermann DK, Groettrup M. Prostate stem cell antigen is a promising candidate for immunotherapy of advanced prostate cancer. *Cancer Res* 2000;60:5522-8.
- Schäkel K, Mayer E, Federle C, Schmitz M, Riethmüller G, Rieber

- EP. A novel dendritic cell population in human blood: one-step immunomagnetic isolation by a specific MAb (M-DC8) and in vitro priming of cytotoxic T lymphocytes. *Eur J Immunol* 1998;28:4084-93.
23. Rammensee H-G, Bachmann J, Emmerich NPN, Bacher OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999;50:213-9.
24. Van der Burg SH, Ras E, Drijfhout JW, Benckhuijsen WE, Bremers AJ, Melief CJ, Kast WM. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. *Hum Immunol* 1995;44:189-98.
25. Herr W, Linn B, Leister N, Wandel E, Meyer zum Büschenfelde K-H, Wölfel T. The use of computer-assisted video image analysis for the quantification of CD8⁺ T lymphocytes producing tumor necrosis factor α spots in response to peptide antigens. *J Immunol Methods* 1997;203:141-52.
26. Janetzki S, Song P, Gupta V, Lewis JJ, Houghton AN. Insect cells as HLA-restricted antigen-presenting cells for the IFN- γ Elispot assay. *J Immunol Methods* 2000;234:1-12.
27. Röpke M, Hald J, Guldberg P, Zeuthen J, Nørgaard L, Fugger L, Sveigaard A, van der Burg S, Nijman HW, Melief CJM, Claesson MH. Spontaneous human squamous cell carcinomas are killed by a human cytotoxic T lymphocyte clone recognizing a wild-type p53-derived peptide. *Proc Natl Acad Sci USA* 1996;93:14704-7.
28. Butterfield LH, Koh A, Meng W, Vollmer CM, Ribas A, Disette V, Lee E, Glaspy JA, McBride WH, Economou JS. Generation of human T-cell responses to an HLA-A2.1-restricted peptide epitope derived from α -fetoprotein. *Cancer Res* 1999;59:3134-42.
29. Andersen MH, Pedersen LØ, Becker JC, Thor Straten P. Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients. *Cancer Res* 2000;61:869-72.
30. Jäger E, Nagata Y, Gnjatich S, Wada H, Stockert E, Karbach J, Dunbar PR, Lee SY, Jungbluth A, Jäger D, Arand M, Ritter G, et al. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci USA* 2000;97:4760-5.
31. Jäger E, Jäger D, Karbach J, Chen Y-T, Ritter G, Nagata Y, Gnjatich S, Stockert E, Arand M, Old LJ, Knuth A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4⁺ T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J Exp Med* 2000;191:625-30.
32. Meidenbauer N, Harris DT, Spittler LE, Whiteside TL. Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. *Prostate* 2000;43:88-100.
33. Letsch A, Keilholz U, Schadendorf D, Nagorsen D, Schmittel A, Thiel E, Scheibenbogen C. High frequencies of circulating melanoma-reactive CD8⁺ T cells in patients with advanced melanoma. *Int J Cancer* 2000;87:659-64.

Advances in Brief

A Phase I Trial of a Recombinant Vaccinia Virus Expressing Prostate-specific Antigen in Advanced Prostate Cancer¹

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Abstract

A recombinant vaccinia virus encoding human prostate-specific antigen (rV-PSA) was administered as three consecutive monthly doses to 33 men with rising PSA levels after radical prostatectomy, radiation therapy, both, or metastatic disease at presentation. Dose levels were 2.65×10^6 , 2.65×10^7 , and 2.65×10^8 plaque forming units. Ten patients who received the highest dose also received 250 $\mu\text{g}/\text{m}^2$ granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immunostimulatory adjunct. No patient experienced any virus-related effects beyond grade I cutaneous toxicity. Pustule formation and/or erythema occurred after the first dose in all 27 men who received $\geq 2.65 \times 10^7$ plaque forming units. GM-CSF administration was associated with fevers and myalgias of grade 2 or lower in 9 of 10 patients. PSA levels in 14 of 33 men treated with rV-PSA with or without GM-CSF were stable for at least 6 months after primary immunization. Nine patients remained stable for 11–25 months; six of these remain progression free with stable PSA levels. Immunological studies demonstrated a specific T-cell response to PSA-3, a 9-mer peptide derived from PSA. rV-PSA is safe and can elicit clinical and immune responses, and certain patients remain without evidence of clinical progression for up to 21 months or longer.

Introduction

Prostate cancer was diagnosed in ~174,500 men in 1998 (1). Thirty percent of patients have metastatic disease at presentation, and of those treated with primary radical prostatectomy or radiation therapy, a significant proportion will relapse. Treatment with androgen ablation for recurrent prostate cancer, although providing effective palliation in many patients, is rarely curative, and the vast majority patients eventually demonstrate progressive disease (2–4). Effective hormonal therapy requires surgical or medical castration with resulting impotence, loss of libido, loss of muscle mass, weight gain, gynecomastia, and hot flashes. Patients with rising PSA³ after primary therapy will inevitably progress to overt metastatic disease. At present, the treatment options for this group of patients with small tumor burdens and otherwise normal health are to wait for the occurrence of overt metastatic disease, to begin hormonal therapy with its attendant side effects, or to enroll in investigational therapies.

Because PSA is expressed exclusively in prostatic epithelial cells, whether normal or transformed, it represents an attractive target for immunotherapy (5). The availability of a recombinant vaccinia virus that expresses rV-PSA allows clinical testing of this concept. Because vaccinia evokes both humoral and cell-mediated responses, co-expression of PSA with viral proteins may enhance immunogenicity to cells expressing PSA, resulting in the lysis of cells expressing this antigen.

CTLs recognize protein antigens as small peptides (9–10 amino acids long) associated with MHC class I molecules (6, 7). Previous studies involved a determination of whether PSA contains epitopes capable of binding class I HLA A2 molecules and could elicit CD8⁺ cytolytic T-lymphocyte responses, and whether the CTLs generated could lyse human prostatic cancer cell lines in a MHC-restricted manner (8, 9). The HLA-A2 allele was chosen for study because it is expressed in ~50% of the population and the HLA binding motifs for peptides are known. Several peptides within the PSA molecule have been identified that bind the A2 site. These peptides, when incubated with PBMCs from normal donors (serving as antigen-presenting cells) in the presence of interleukin-2, were able to generate T-cell lines. Several of these T-cell lines when pulsed with one of these peptides, designated PSA-3, were able to lyse HLA-A2-positive target cell lines and cell lines infected with rV-PSA but not wild-type vaccinia-infected cells. More importantly, these T-cell lines were able to lyse human prostatic carcinoma cell lines and other cell types expressing PSA and possessing the HLA-A2 allele (8). Subsequent studies have identified other

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³ The abbreviations used are: PSA, prostate-specific antigen; rV-PSA, recombinant vaccinia virus expressing PSA; PBMC, peripheral blood mononuclear cell; pfu, plaque-forming unit(s); GM-CSF, granulocyte/macrophage-colony stimulating factor; CEA, carcinoembryonic antigen; DLT, dose-limiting toxicity; Flu, influenza.

epitopes of PSA capable of eliciting CTLs that will lyse PSA expressing prostate carcinoma cells (9).

Recombinant vaccinia virus constructs expressing tumor-associated antigens have been developed and tested in primates and humans without significant toxicity (10–12). In a preclinical toxicology study, rV-PSA was administered to rhesus monkeys, which have a 94% amino acid homology to human PSA. Local erythema, regional lymphadenopathy, and transient elevations of WBC counts were observed. Mild temperature elevations were seen in some animals. There was no effect on body weight, clinical chemistries, or other untoward effects. A PSA-specific IgM response was seen in all monkeys tested, and all four of the monkeys inoculated with 1×10^8 pfu rV-PSA developed specific T-cell responses to PSA protein for up to 270 days (13).

Several biological adjuvants have been used to enhance T-cell responses to tumor-associated antigens. One of the more promising of these adjuvants is recombinant GM-CSF (14–16). When administered either at the site of a peptide immunization in experimental systems or with an anti-idiotypic monoclonal antibody protein in clinical studies, GM-CSF was shown to enhance antigen-specific immune responses (14, 15). In recent experimental studies, GM-CSF was shown to enhance the immune response to CEA when given the day of and 3 consecutive days after a rV-CEA vaccine (17).

We have conducted a phase I trial of rV-PSA vaccine in men with advanced cancer of the prostate. We report here that administration of rV-PSA is safe and that the vaccine induces PSA-specific immune responses and potential clinical activity.

Patients and Methods

Patient Selection

Patients were required to have a histologically confirmed diagnosis of prostatic adenocarcinoma and evidence of advanced disease by any of the following: (a) lymph node involvement with cancer and PSA ≥ 10 ng/ml; (b) bone scan representing metastatic cancer and PSA ≥ 10 ng/ml; (c) post radical prostatectomy with rising PSA ≥ 2 ng/ml (three determinations monthly or greater); or (d) post radiation therapy and PSA ≥ 10 ng/ml and rising. Patients needed to be Eastern Cooperative Oncology Group performance status 0 or 1; have adequate hematological, hepatic, and renal function; have normal immunological testing as defined by positive delayed hypersensitivity skin testing (mumps, *Candida*, and trichophyton); have a CD4:CD8 ratio > 1 ; and have normal serum immunoglobulin levels. Prior vaccinia exposure (as the small pox vaccine in childhood or during military service) was required, either by documentation, presence of an inoculation scar, or patient history. Neoadjuvant hormonal therapy was permitted as long as the preceding criteria for prostate cancer progression were met.

Exclusion criteria were prior hormonal therapy or chemotherapy for advanced disease, a history of autoimmune diseases associated with altered immune function, and prior splenectomy or active cases of skin disorders, such as eczema, extensive psoriasis, disseminated zoster, burns, or impetigo. Evidence of metastatic bony disease, including bony pain or radionuclide imaging, was an exclusion.

Treatment Plan

rV-PSA vaccinations were administered to each patient at 4-week intervals for a total of three doses. Three dose levels were used. Six patients were treated at the lowest dose level and evaluated for toxicity at 4 weeks after initial vaccination before they were entered at the next higher dose level. Patients were followed weekly until 28 days after the final dose (day 85) and then monthly for 6 months. When the highest dose of the rV-PSA vaccine was reached, an additional five patients were treated to further assess potential toxicity. An additional 10 patients were treated with GM-CSF in conjunction with the rV-PSA at dose level of 2.65×10^8 pfu. GM-CSF (250 μ g/m²) was administered s.c. on days -1, 0, 1, and 2 of the rV-PSA treatment. The intradermal rV-PSA was administered in the skin immediately above the s.c. GM-CSF site. If patients experienced nonhematological toxicity of grade 3 or higher, the GM-CSF dose was reduced to 125 μ g/m².

Patients were seen weekly for the first 2 months, and then monthly. Complete interval histories, physical examinations, blood chemistries, electrocardiograms, and serum PSA were obtained. All patients were evaluated for toxicity by the Common Toxicity Criteria 2.0 (<http://www.ctep.nci.nih.gov>) and the vaccinia toxicity grading scale (Centers for Disease Control). Patients were followed until disease progression.

DLT was defined as any of the following: grade 4 vaccinia toxicity; grade 4 hematological toxicity; or grade 3/4 nonhematological toxicity, except nausea, vomiting, or fever. The maximum tolerated dose was defined as the dose below that dose at which DLT occurred in two patients.

Vaccinia-related Toxicity Grading. Vaccinia-related toxicity grading was as follows: grade 1, cutaneous reaction extending ≤ 10 cm from the vaccination site; grade 2, generalized cutaneous reaction extending > 10 cm from the vaccination site, and autoinoculation syndrome without sequelae; grade 3 was not applicable; grade 4, autoinoculation syndrome with sequelae; post vaccinia encephalitis, vaccinia gangrenosum, eczema gangrenosum, and Stevens-Johnson syndrome (18).

Criteria for Response. No patient on this trial had measurable disease. Therefore, the following response criteria were used: complete response was defined as normalization of the PSA value for three successive monthly determinations; a partial response was defined as a decline of PSA value by $> 80\%$ (without normalization) for three successive monthly determinations; stable disease was defined as a decline in PSA of $< 80\%$ or an increase in PSA value up to 50% for three successive monthly determinations; and progressive disease was defined as any increase in PSA to $> 50\%$ above baseline for three successive monthly determinations or the appearance of new lesions.

Vaccine Formulation

rV-PSA, constructed and manufactured by Therion Biologicals Corporation, was provided by the NCI (NSC no. 678892). The vaccine was derived from the Wyeth [New York City Board of Health, associated with the lowest incidence of clinical complications (19)] strain of vaccinia by the insertion of the PSA gene into the viral genome (13). Two equivalent lots of rV-PSA were used during the study. The first lot included vials containing 0.1 ml of vaccine at a concentration of 2.65×10^9 pfu/ml in PBS with 10% glycerol. In the second lot, vaccine

Table 1 Toxicity

Group	Treatment	Toxicity (number of patients/total patients)		
		Alkaline phosphatase	Fever ^a	Cardiovascular
1	2.65×10^6 pfu rV-PSA	0/6	0/6	0/6
2	2.65×10^7 pfu rV-PSA	0/6	0/6	0/6
3	2.65×10^8 pfu rV-PSA	1/11	0/11	0/11
4	2.65×10^8 pfu rV-PSA + 250 $\mu\text{g}/\text{m}^2$ GM-CSF	0/10	1/10	1/10

^a Grades 2 and 3.

vials contained 0.3 ml at a concentration of 1.17×10^9 pfu/ml. Administration was via intradermal inoculation using a needle and syringe. A sterile, nonadherent dressing (*i.e.*, "Telfa") was used to cover the site.

Collection of PBMCs

PBMCs were collected in heparinized tubes from HLA-A2-positive patients treated with rV-PSA and GM-CSF. The mononuclear cell fraction was separated by Ficoll-Hypaque density gradient separation, washed three times with cold PBS, and frozen in 90% heat-inactivated AB serum and 10% DMSO at -80°C until assayed. Five vials per patient were stored, with 1×10^7 cells/vial. After collection of serial samples, each patient's pretreatment, postvaccination-1, post-2, and post-3 samples were resuspended in RPMI 1640 supplemented with 15 mM HEPES buffer (pH 7.4), 10% pooled, heat-inactivated AB serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (20).

Immunoassay

PBMCs from pre, post-1, post-2, and post-3 immunizations were thawed and used as effector cells. PBMCs were seeded at a concentration of 2×10^5 /well in six wells. Cells were cultured for 24 h in the presence of PSA-3-pulsed C1R-A2 cells (stimulator:effector ratio = 1:5) and measured for IFN- γ release using the ELISPOT assay (21). Flu peptide 58-66 was used as a control. PBMCs from a Flu peptide-responsive donor were also used as a positive internal control. The PSA-3 and the Flu 58-66 peptides were prepared from a peptide synthesizer as described (8).

Anti-PSA Antibody ELISA Assay

The presence of anti-PSA antibodies in patient serum pre and post vaccination was analyzed using an ELISA. Polyvinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated overnight at 4°C with a purified preparation of PSA (Vitro Diagnostics, Littleton, CO), as well as BSA or human serum albumin at 100 ng/well in 50 μl of PBS (pH 7.2). The wells were blocked for 1 h with PBS containing 1% BSA (assay buffer). Patient serum and control pooled human serum (Gemini Bioproducts, Calabasas, CA) were diluted in assay buffer and added to wells in triplicate in a volume of 50 μl /well. Purified human antimurine PSA-specific IgG antibody (Fitzgerald Industries, Concord, MA) was used as a positive control for PSA binding. Purified human IgG (Jackson ImmunoResearch, West Grove, PA) was used as a negative control.

After incubation overnight at room temperature, the wells were washed four times with assay buffer, and 50 μl of a 1:4000 dilution of peroxidase-conjugated goat antihuman IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to each well. A 1:2000 dilution of peroxidase-conjugated goat antimurine IgG (Kirkegaard & Perry) was used for the PSA antibody control. After incubation at 37°C for 1 h, wells were washed four times with assay buffer, and 100 μl each of the chromogen *O*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and hydrogen peroxide were added to each well. After a 10-min incubation in the dark, the reaction was stopped with 25 μl of 4N NH_2SO_4 . The absorbance of each well was measured at 490 nm using an ELISA microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Results

Safety. Thirty-three men were treated over a 22-month period. The median age was 62 years (range, 47–74 years). No DLT occurred, and no maximum tolerated dose was reached due to the formulation potency of rV-PSA. At the highest dose, 2.65×10^8 pfu, treatment with 250 $\mu\text{g}/\text{m}^2$ of GM-CSF was added.

The toxicities observed are shown in Table 1. No vaccinia-related toxicity higher than grade 1, a local reaction at the site of inoculation, occurred in any patient. There was no lymphadenopathy, hepatosplenomegaly, fever, malaise, or leukocytosis attributable to the recombinant vaccine. In the 10 patients treated with GM-CSF, 1 patient developed grade 3 fever and tachycardia after the first dose of GM-CSF, prior to rV-PSA administration, and required a dose reduction to 125 $\mu\text{g}/\text{m}^2$. This dose was tolerated without further symptoms.

The cutaneous responsiveness to the first and subsequent rV-PSA vaccinations is listed in Table 2. Thirty-two of 33 patients responded locally to the first administration of rV-PSA. At doses $\geq 2.65 \times 10^7$ pfu, the majority of patients exhibited local responses even after the third vaccination (not all patients were assessed after the third inoculation for a cutaneous reaction).

Immune Responses to PSA. T-cell assays using the HLA-A2 class I allele 9-mer peptides PSA-3 and Flu matrix were used to investigate T-cell responses in patients positive for the HLA-A2 allele in the cohort of patients treated with 2.65×10^8 pfu rV-PSA plus GM-CSF. Seven patients were positive for the class I HLA-A2 allele. Ficoll-purified PBMCs from each of these patients were viably frozen at 1×10^7 cells/ml. PBMCs were obtained pre vaccination and 1 month after each vaccination for each patient. ELISPOT assays using the PSA-3 and Flu

Table 2 Number of patients exhibiting cutaneous response^a after each immunization

Group	No. patients	Treatment	Dose 1	Dose 2	Dose 3 ^b
1	6	2.65×10^6 pfu	5/6	2/6	0/6
2	6	2.65×10^7 pfu	6/6	6/6	4/5
3	11	2.65×10^8 pfu	11/11	7/10	3/4
4	10	2.65×10^8 pfu + 250 $\mu\text{g}/\text{m}^2$ GM-CSF	10/10	10/10	7/10

^a Erythema >1 with or without vesicle formation.^b Not all patients assessed.

peptides were done simultaneously and coded. PBMCs were assayed after only 24 h in culture in the presence of peptide to rule out the effects of *in vitro* selection of T-cell populations. Results are expressed as a precursor frequency of IFN- γ -secreting cells in response to the given peptide.

As listed in Table 3, responses to the Flu peptide were similar pre vaccination and after each of three vaccinations with rV-PSA. These data served as an internal control for the ELISPOT assay using the PSA-3 peptide. Increases of at least 2-fold in precursors specific for PSA-3 peptide were observed in five of seven patients after three vaccinations, with patient 33 showing a >4.6-fold increase. In these five patients, the greatest increase in PSA-specific precursor frequency was observed after the first vaccination. Subsequent vaccinations did not produce substantial additional increases.

All 33 patients were tested for IgM and IgG antibodies to PSA. One patient (patient 24) developed low-level IgG antibodies to PSA. No other patient tested positive for anti-PSA antibodies (data not shown).

Clinical/Serum PSA Responses. Fig. 1 shows the number of months that each patient exhibited stable PSA levels after primary vaccination. PSA levels in 14 of 33 men treated with rV-PSA with or without GM-CSF were stable for at least 6 months after the initial immunization. Nine patients remained stable for 11–25 months. Six patients remain on study with stable PSA levels. Two patients had elevated lactate dehydrogenase levels at the time of initial enrollment, which remained elevated after vaccination. The progression-free interval for these patients ranged from 11+ to 21+ months. In addition to continuing to exhibit stable PSA levels, the six patients remaining on study showed no signs or symptoms of prostate cancer, including restaging bone scans.

Nineteen of 33 patients showed a decrease in PSA levels at some time during the treatment period. This transient decrease was not associated with detectable IgG antibody formation to PSA (data not shown). Several patients developed T-cell immune responses associated with prolonged stabilization.

Discussion

PSA is a 34-kDa glycoprotein produced by prostatic epithelial cells lining the glandular acini and ducts (1, 22). PSA is a serine protease of the glandular kallikrein gene family, which when secreted into prostatic and seminal fluid hydrolyzes seminal vesicle proteins important in semen liquefaction. Although present in the serum of healthy males at low levels, elevated PSA levels are correlated with prostatic epithelial growth, being elevated in 40% of men with benign prostatic hypertrophy. Men

Table 3 ELISPOT assay for IFN- γ production in PBMCs of patients pre- and post vaccinations with rV-PSA (plus GM-CSF)

Results are expressed as precursor frequency of IFN- γ -secreting cells. PBMCs from pre, post-1, post-2, and post-3 vaccinations were used as effector cells. PBMCs were seeded at a concentration of 2×10^5 /well in six wells. Cells were cultured for 24 h in the presence of PSA-3-pulsed C1R-A2 cells. Flu matrix peptide 58–66 was used as control. A higher number of precursors is expressed as a smaller number in the denominator of the precursor frequency. Increases in precursor frequencies ≥ 2 -fold were considered positive. Statistically significant difference ($P = 0.0313$, univariate procedure using the SAS system) for the following comparison: PSA-3 (pre vs. post). No statistically significant difference ($P = 1.000$, univariate procedure using the SAS system) for comparison with Flu peptide 58–66 (pre vs. post).

Patient	Sample	Peptide	
		Flu 58–66	PSA-3
024	Pre	1/24,000	1/54,546
	Post-1	1/21,428	1/35,294
	Post-2	1/19,230	1/22,727
	Post-3	1/26,087	1/27,273
027	Pre	1/11,538	1/11,765
	Post-1	1/11,538	1/13,043
	Post-2	1/9,538	1/10,526
	Post-3	1/12,500	1/10,169
028	Pre	1/22,222	1/31,579
	Post-1	1/43,857	1/33,333
	Post-2	1/120,000	1/100,000
	Post-3	1/33,333	1/37,500
029	Pre	1/100,000	1/150,000
	Post-1	1/100,000	1/66,666
	Post-2	1/120,000	1/60,000
	Post-3	1/120,000	1/75,000
030	Pre	1/42,857	1/75,000
	Post-1	1/33,333	1/42,857
	Post-2	1/37,500	1/31,000
	Post-3	1/33,333	1/33,333
032	Pre	1/40,000	1/85,714
	Post-1	1/40,000	1/37,500
	Post-2	1/42,000	1/33,333
	Post-3	1/40,000	1/40,000
033	Pre	1/37,500	<1/200,000
	Post-1	1/60,000	1/40,000
	Post-2	1/37,000	1/40,000
	Post-3	1/33,333	1/42,857

with prostate cancer show exponential increases in circulating levels of PSA (23). PSA is expressed in the majority of primary and metastatic prostate cancers, making it a target for cancer immunotherapy.

In vitro studies have demonstrated the generation of human CTLs specific for peptides derived from PSA (15). PSA-1 and

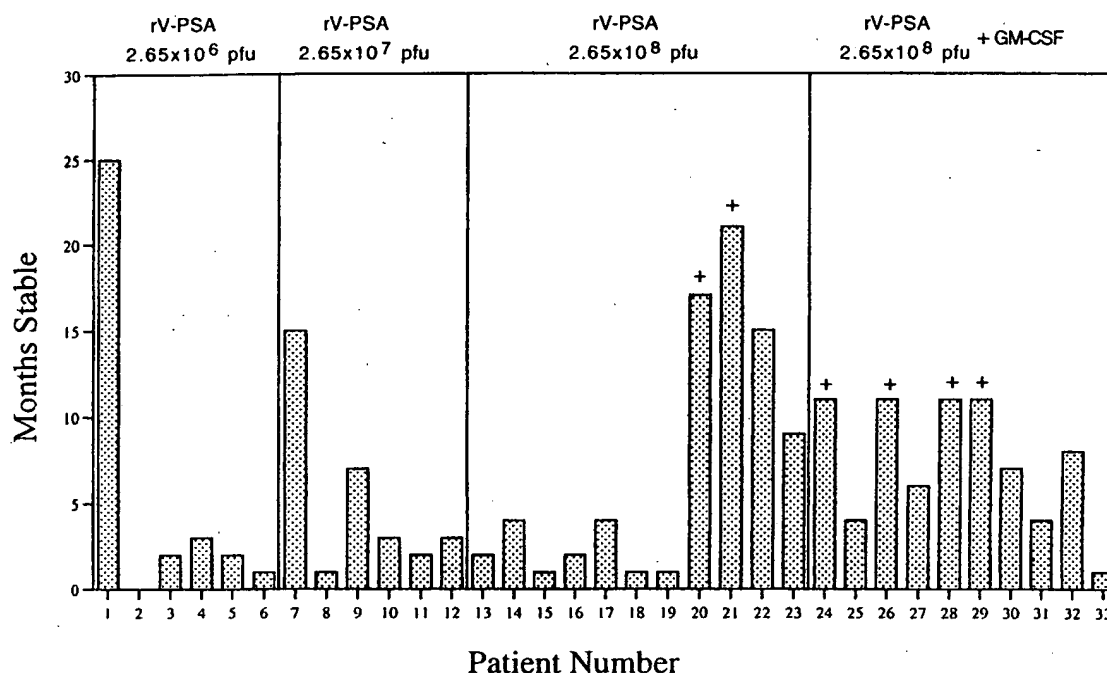


Fig. 1 Number of months that each patient exhibited stable PSA levels after primary vaccination. Stable PSA levels are defined as a decline of <80% or an increase in PSA levels up to 50%. + indicates that PSA levels remained stable in that patient.

PSA-3, peptides with HLA-A2 binding motifs of 10 amino acids in length, produced CTLs from the PBMCs of normal human donors capable of lysing HLA-A2-positive CIR-A2 cells pulsed with these peptides or HLA-matched prostatic carcinoma cell lines. Inoculation of human HLA-A2/K^b transgenic mice with a PSA peptide produced CTLs against PSA-expressing, HLA-A2-restricted cell lines. PSA-specific CTL clones demonstrated cytolytic activity against HLA-A2-positive CIR-A2 cell lines infected with rV-PSA but not against the same leukemic cell lines infected with wild-type vaccinia only, further demonstrating that human cells can process PSA so that PSA-peptide MHC complexes on cell surfaces make that cell susceptible to CTL-mediated lysis (16). PSA can be considered a potential target for cell-mediated immunotherapy. This would be particularly applicable in circumstances where prostate ablation by prostatectomy or radiotherapy has been performed and a rising PSA would be indicative of metastatic disease.

Vaccinia virus is a DNA orthopoxvirus with a large genome that serves well as an expression vector for large foreign proteins (24, 25). Unlike other DNA viruses, vaccinia replicates in the host cell cytoplasm. Under the regulation of a vaccinia promoter, the inserted foreign genes are transcribed and translated, and the resultant proteins are processed and transported (26). Inoculation of epidermal keratinocytes by scarification, intradermal, or s.c. routes produces a localized infection that allows expression of the foreign gene in host cells, and with virus multiplication, there is amplification of antigen with greater potential for immune reactivity. Both humoral and cell-mediated immunity can be elicited toward the foreign gene product (27, 28).

Preclinical safety trials in rhesus monkeys demonstrated

that rV-PSA is safe and capable of inducing PSA-specific immune responses in a dose-dependent fashion. No evidence of autoimmunity or histopathology was observed. Three clinical trials using recombinant vaccinia vectors encoding CEA (rV-CEA) have demonstrated local vaccination site erythema, vesicular/pustular reactions, regional node swelling, malaise, and fevers without any other toxicity (6–8), as well as the generation of CEA-specific T-cell responses (13).

This phase I trial of rV-PSA demonstrated that repeated vaccination with rV-PSA is safe and nontoxic in men with prostate cancer. The addition of GM-CSF increased the incidence and severity of toxicity, but all of the toxicities were deemed a consequence of cytokine therapy and were mild in all but one circumstance. Cutaneous responses indicative of viral replication occurred in 32 of 33 patients despite previous exposure to vaccinia virus as a smallpox vaccine. Other than a local dermal reaction, there was no vaccinia-related toxicity.

Recombinant GM-CSF was used in these studies because previous preclinical and clinical studies using peptide or protein as immunogen showed that GM-CSF enhanced T-cell responses (14–16). In one of these studies in a rat model (14), the optimal dose and schedule was to administer GM-CSF at the injection site the day of vaccination. A recent study in a murine model not only confirmed that this dose schedule was optimal, but also demonstrated that it enhanced the CEA-specific T-cell responses when a recombinant vaccinia virus was used as immunogen (17). These studies also showed that GM-CSF enhanced the infiltration of dendritic cells to regional lymph nodes (17).

A recently reported limited phase I clinical trial evaluated the safety and biological effects of rV-PSA administered in six

patients with androgen-modulated recurrence of prostate cancer after radical prostatectomy. Toxicity was minimal, and DLT was not observed. Noteworthy variability in the time required for androgen restoration (after interruption of androgen deprivation therapy) was observed. Primary anti-PSA IgG antibody activity was induced after rV-PSA vaccination in one patient. One patient had a prolonged period with undetectable serum PSA levels after androgen restoration (29).

Seven HLA-A2 positive patients treated with 2.65×10^8 pfu rV-PSA and GM-CSF in our trial had PBMCs assayed for the development of a PSA peptide-specific T-cell immune response. Increases of at least 2-fold in precursors specific for the PSA-3 peptide were observed in five of seven patients after three vaccination, with one patient showing a 4.6-fold increase, whereas precursor frequencies specific for the control Flu peptide never increased more than 0.2-fold in any patient tested. In four of these five patients with an increased PSA-specific immune response, there was stabilization of serum PSA levels for 6–11+ months.

Stabilization of serum PSA has continued in 6 of 33 patients for 11+ to 21+ months. Several other patients were observed to have stabilization in the serum PSA levels for periods up to 2 years. The doubling time of the PSA for 6 months prior to vaccination with rV-PSA as opposed to that in 6-month intervals after vaccination suggests a change in the course of the disease in some patients. The PSA response correlated with the lack of any other marker of disease progression in these patients. The rise in PSA over time suggests that immunity needs boosting at periodic intervals.

The studies reported here demonstrated that rV-PSA vaccination enhanced T-cell responses to PSA after the first vaccination only, as opposed to the second or third. This is most likely due to host-immune responses to vaccinia proteins that limit the replication of the vaccinia virus. All of the patients in this study had received a small pox vaccination in childhood and most again during military service, thus enhancing the probability of a strong immune response to vaccinia proteins. These findings, along with preclinical observations (28), suggest that rV-PSA is best used in priming the immune system to a weak antigen such as PSA, and that another immunogen be used to boost the immune response.

These stabilizations in PSA concentrations were not correlated with the formation of serum IgM or IgG antibodies against PSA; no detectable antibodies were found in 32 of 33 men pre or post vaccination. One patient with stable PSA levels demonstrated production of a low-level titer of antibody to PSA. The inability to detect antibodies to PSA does not necessarily mean that none were generated. It is possible that antibodies could bind circulating PSA and be rapidly cleared by reticuloendothelial cells. Antibodies could also bind to tumor-associated PSA, although PSA is a predominantly secreted protein. In either circumstance, the titer of antibody would be sufficiently low that serum PSA was still readily detectable because an absolute decrease in PSA was infrequent.

This study demonstrates for the first time that PSA can serve as a target for cell-mediated immunotherapy approaches. Prostate cancer patients who expressed elevated serum PSA levels in the absence of symptomatic metastatic involvement

tolerated repeated vaccinations with rV-PSA in doses of 2.65×10^8 pfu, and certain patients demonstrated PSA peptide-specific cellular responses. A subset of patients have had stabilization of serum PSA levels in the absence of clinical signs of disease progression for up to 25 months. The eventual rise in PSA levels in most patients in this phase I study suggests that immune responses elicited by rV-PSA require boosting. Preclinical studies with recombinant poxviruses expressing CEA have suggested that priming with recombinant vaccinia virus and boosting with recombinant avipox virus elicited greater CEA-specific T-cell responses than with either vector alone (28). This observation is further supported by recent human clinical studies evaluating prime-boost regimens with recombinant vaccinia and avipox viruses expressing CEA (30). These results, combined with the study reported here, have led to the initiation of a phase II clinical trial using a prime and boost administration of rV-PSA and recombinant avipox (fowlpox) virus expressing PSA in patients with prostate cancer.

References

1. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1996. *CA Cancer J. Clin.*, 46: 5–27, 1996.
2. Blackhard, C. E. The Veteran's Administration Cooperative Urology Group studies of carcinoma of the prostate: a review. *Cancer Chemother. Rep.*, 59: 225–231, 1975.
3. Byar, D. P., and Corle, D. Hormone therapy for prostate cancer: results of the Veteran's Administration Cooperative Urology Group studies. *In: Consensus Development Conference in the Management of Clinically Localized Prostate Cancer. Natl. Cancer Inst. Monogr.*, 7: 165–172, 1988.
4. Von Tinter, H., and Dalesio, O. Systematic overview (metaanalysis) of all randomized trials in the treatment of prostate cancer. *Cancer (Phila.)*, 72(Suppl.): 3847–3850, 1993.
5. Wang, M. C., Valenzuela, L. A., Murphy, G. P., and Chu, T. M. Purification of prostate specific antigens. *Investig. Urol.*, 17: 159–163, 1979.
6. Wolfel, T., Klehman, E., Muller, C., Meyer zum Buschenfelde, K. H., Schutt, K. H., and Knuth, A. Lysis of human melanoma cells by autologous cytolytic T cell clones. Identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. *J. Exp. Med.*, 170: 797–810, 1989.
7. Crowley, N. J., Darrow, T. L., Quinn-Allen, M. A., and Seigler, H. F. MHC-restricted recognition of autologous melanoma by tumor specific cytotoxic T cells. *J. Immunol.*, 146: 1692–1699, 1991.
8. Correale, P., Walmsley, K., Nieroda, C., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Y. *In vitro* generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. *J. Natl. Cancer Inst.*, 89: 293–300, 1997.
9. Correale, P., Walmsley, K., Nieroda, C., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Generation of human cytolytic T lymphocyte lines directed against prostate specific antigen (PSA) employing a PSA oligopeptide peptide. *J. Immunol.*, 161: 3186–3194, 1998.
10. Hamilton, J. M., Chen, A. P., Nguyen, B., Grem, J., Abrams, S., Chung, Y., Kantor, J., Phares, J. C., Bastien, A., and Brooks, C. A phase I study of recombinant vaccinia virus that expresses human carcinoembryonic antigen (CEA) in adult patients with adenocarcinoma. *Proc. Am. Soc. Clin. Oncol.*, 13: 295, 1995.
11. McAneny, D., Ryan, C. A., Beazley, R. M., and Kaufman, H. L. The results of a phase I trial of a recombinant vaccinia virus that expresses carcinoembryonic antigen in patients with advanced colorectal cancer. *Ann. Surg. Oncol.*, 3: 495–500, 1996.
12. Conroy, R. M., Saleh, M. N., Schlom, J., and LoBuglio, A. F. Breaking tolerance to carcinoembryonic antigen with a recombinant

- vaccinia virus vaccine in man. *Proc. Am. Assoc. Cancer Res.*, 36: 2930, 1995.
13. Hodge, J. W., Schlom, J., Donohue, S. J., Tomaszewski, J. E., Wheeler, C. W., Levine, B. S., Gritz, L., Panicali, D., and Kantor, J. A. A recombinant vaccinia virus expressing human prostate-specific antigen (PSA): safety and immunogenicity in a nonhuman primate. *Int. J. Cancer*, 63: 231-237, 1995.
 14. Disis, M. L., Bernhard, H., Shiota, F. M., Hand, S. L., Gralow, J. R., Huseby, E. S., Gilles, S., and Cheever, M. A. Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines. *Blood*, 88: 202-210, 1996.
 15. Chen, T. T., Tao, M.-H., and Levy, R. Idiotypic-cytokine fusion proteins as cancer vaccines. Relative efficiency of IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor. *J. Immunol.*, 153: 4775-4787, 1994.
 16. Kwak, L. W., Young, H. A., Pennington, R. W., and Weeks, S. D. Vaccination with syngeneic, lymphoma-derived immunoglobulin idiotype combined with granulocyte-macrophage colony-stimulating factor primes mice for a protective T-cell response. *Proc. Natl. Acad. Sci. USA*, 93: 10972-10977, 1996.
 17. Kass, E., Parker, J., Schlom, J., and Greiner, J. W. Comparative studies of the effects of recombinant GM-CSF administered via a poxvirus to enhance the concentration of antigen presenting cells in regional lymph nodes. *Cytokine*, in press, 2000.
 18. Centers for Disease Control. Vaccinia (smallpox) vaccine recommendation of the Immunization Practices Advisory Committee (ACIP). *Morb. Mortal. Wkly. Rep.*, 40: 1-10, 1991.
 19. Fenner, F., Henderson, D. A., and Arita, L. Vaccines in Smallpox, and its Eradication. Geneva: WHO, 1988.
 20. Tsang, K. Y., Zhu, M. Z., Nieroda, C. A., Zaremba, S., Hamilton, J. M., Cole, D., Lam, C., and Schlom, J. Phenotypic stability of a cytotoxic T cell line directed against an immunodominant epitope of human carcinoembryonic antigen. *Clin. Cancer Res.*, 3: 2439-2449, 1997.
 21. Scheibenbogen, C., Kang-Hun, L., Mayer, S., Stevanovic, S., Moebius, U., Herr, W., Rammensee, H., and Keilholz, U. A sensitive ELISPOT assay for the detection on CD8+ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. *Clin. Cancer Res.*, 3: 221-226, 1997.
 22. Bilhartz, D. L., Tindall, D. J., and Oesterling, J. E. Prostate-specific antigen and prostatic acid phosphatase; biomolecular and physiologic characteristics. *Urology*, 38: 95-102, 1991.
 23. Karr, J. F., Kantor, J. A., Hand, P. H., Eggensperger, D. L., and Schlom, J. The presence of prostate-specific antigen genes in primates and expression of recombinant human prostate-specific antigen in a transfected murine cell line. *Cancer Res.*, 55: 2455-2462, 1995.
 24. Mackett, M., Smith, G. L., and Moss, B. General method for production and selection of vaccinia virus recombinants expressing foreign genes. *J. Virol.*, 49: 857-864, 1984.
 25. Moss, B. Vaccinia virus: a tool for research and vaccine development. *Science (Washington DC)*, 252: 1662-1667, 1991.
 26. Moss, B., Smith, G. L., Gerin, J. L., and Purcell, R. H. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. *Nature (Lond.)*, 311: 67-69, 1984.
 27. Bernards, R., Desree, A., McKenzie, S., Gordon, E., Weinberg, R. A., and Panicali, D. Effective tumor immunotherapy directed against an oncogene-encoded product using vaccinia virus vector. *Proc. Natl. Acad. Sci. USA*, 84: 6854-6858, 1987.
 28. Hodge, J. W., McLaughlin, J. P., Kantor, J. A., and Schlom, J. Diversified prime and boost protocols using recombinant vaccinia virus and recombinant nonreplicating avian pox virus to enhance T-cell immunity and antitumor responses. *Vaccine*, 16: 759-768, 1997.
 29. Sanda, M. G., Smith, D. C., Charles, L. G., Hwang, C., Pienta, K. J., Schlom, J., Milenic, D., Panicali, D., and Montie, J. E. Recombinant Vaccinia-PSA (ProstVac) can induce a prostate-specific immune response in androgen-modulated human prostate cancer. *Urology*, 53: 260-266, 1999.
 30. Marshall, J. M., Richmond, E., Pedicano, J. E., Tsang, A., Arlen, P., and Schlom, J. Phase I/II trial of Vaccinia-Cea, and Alvac-Cea in patients with advanced Cea-bearing tumors. *Proc. Am. Soc. Clin. Oncol.*, 18: 438a, 1999.

Dendritic Cell-Based Immunotherapy for Prostate Cancer

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Introduction

Prostate cancer is the most common non-skin malignancy currently diagnosed in American men, second only to lung cancer as the leading cause of cancer death among males.¹ Although the majority of prostate cancer cases are localized to the prostate, nearly a third of newly diagnosed patients has locally advanced or metastatic prostate cancer. Available treatments for metastatic prostate cancer, including hormonal, chemotherapeutic, and radiation strategies, have failed to demonstrate curative potential in these patients.²

Current standard therapies for early-stage, localized prostate cancers—prostatectomy and radiation therapy—are associated with failure rates of nearly 20%.² As a result, increasing numbers of treated-patients develop metastatic disease or remain at very high risk for the development of such disease. Because the options for these primary treatment failures, as with the incident metastatic cases, are few in number and severely limited in

terms of efficacy, there is great demand for new and improved treatments for prostate cancer. Immunotherapeutic approaches to cancer treatment have shown great promise in experimental studies, and a number of immunotherapies are now being tested in clinical settings.

Cancer Immunotherapy Strategies

Tumor suppression phenomena ostensibly mediated by host immune systems have been reported by a number of investigators. These phenomena include spontaneous regression of various tumors; the observation of a direct association between immunosuppression and increased incidence of cancer; and the presence of leukocytes surrounding and infiltrating tumor tissues.³⁻⁵ Expanding knowledge about the specific mechanisms involved in the immune response has been utilized to elicit or amplify anti-tumor immune responses.

T-CELL ACTIVATION

T cell immune responses, for example, are triggered by the interaction of the T cell receptor (TCR) and antigenic peptides bound to major histocompatibility complex (MHC) antigens expressed on the surface of specialized antigen presenting cells (APC).⁶ If this interaction is accompanied by binding of costimulatory receptors (e.g., CD28) to their ligands (CD80 and CD86), then an intracellular cascade of biochemical events is initiated that results in T cell activation and proliferation.^{7,8} Activated T cells, in turn, are able to lyse cells that express the stimulating antigen and the appropriate MHC antigen, in this case, tumor cells.

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CYTOKINES

Additionally, the presence or absence of cytokines, such as interleukins 2, 4, 10, and 12⁹ can have a profound effect on the activation and proliferation of T cells and consequently on the potency and character of the immune response. Cytokines also affect, both positively and negatively, the function of APC.

Based on these observations, interleukin 2 (IL-2), interferons, and granulocyte-macrophage colony stimulating factor (GM-CSF) have been administered systemically to stimulate anti-tumor immune reactivity.¹⁰ IL-2 has also been used *ex vivo* to generate lymphokine activated killer (LAK) cells and, later, to grow tumor infiltrating lymphocytes (TIL) for reinfusion into patients.^{11,12} These forms of adoptive immunotherapy have had limited success in patients with metastatic melanoma,¹² and continue to be refined by methods that increase the proportion of tumor-specific lymphocytes.

VACCINES

An alternative to systemic cytokines and adoptive immunotherapy is vaccination of patients to elicit *in vivo* activation of tumor-specific T cells. Irradiated tumor cells derived from the patient (autologous) or from other individuals (allogeneic) have been utilized to inoculate cancer patients with the aim of generating a therapeutic immune reaction.¹³ Some procedures have introduced genes coding for cytokine or costimulatory molecules into the tumor cells prior to inoculation to enhance recognition by the immune system.^{13,14} Others have isolated specific tumor antigens to focus the immune response on proteins overexpressed by or unique to the tumor.¹⁵

The most recent advance in cancer vaccines has been the use of autologous APC to present tumor antigens to patients' T cells.¹⁶ The rationale for this technique is to use the cells that are specialized for antigen presentation, so that all factors necessary for initiation of the immune response, including those not yet defined, will be present.

Dendritic Cell-Based Immunotherapy

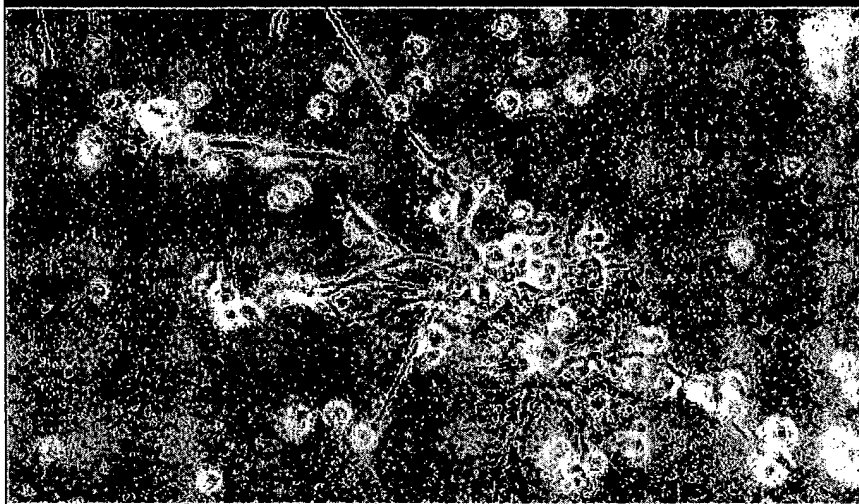
Dendritic cells (DC) (Fig. 1) are considered the most potent APC of the immune system, and are unique in their ability to stimulate naïve T cells.¹⁶ DC are adapted to capture proteins, proteolytically digest them, and present the resulting peptides on their cell membranes bound to MHC antigens.¹⁶ Formation of this MHC-peptide complex is crucial to the activation of T cells. In addition, DC express high levels of the costimulatory molecules, CD80 and CD86, which are required for full T cell activation.¹⁶ DC are found in the epidermal layer of the skin, the respiratory and gastrointestinal systems, and the interstitial regions of several solid organs where they function as sentinels, capturing invading microorganisms for presentation to immune cells.¹⁷ Until recently, the study of DC was limited because few cells could be isolated from tissues or peripheral blood.¹⁸ With improvements in DC isolation and culture techniques, much larger numbers of DC are available and immunotherapy using DC is now feasible. DC can be derived from peripheral blood using cytokines, such as GM-CSF, IL-4, and tumor necrosis factor- α (TNF α).^{19,22}

DC-based cancer vaccines have recently been tested with some success in clinical trials with several types of cancers, such as follicular-B cell lymphoma and melanoma.²³⁻²⁵ Our group has been developing a prostate cancer vaccine using autologous DC as a vehicle to present prostate antigens to T cells *in vivo*. Our phase I and II clinical trials in prostate cancer with DC pulsed with HLA-A0201-specific prostate-specific membrane antigen (PSMA) peptides will be discussed in this report.

Prostate Cancer Vaccine

Two major components comprise our new prostate cancer vaccine. The first component is DC, which are isolated and cultured from patient peripheral blood.

Figure 1
Morphology of Dendritic Cells



A cluster of adherent dendritic cells is shown (320x) in the presence of lymphocytes (round cells).

The second component is a specific antigen used to target prostate cancer tissues. Few antigens specific to prostate tissue have been characterized for use as target antigens for immunotherapy. This short list includes prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA).^{26,27}

PSMA is a 750 amino-acid membrane-bound protein expressed by prostate epithelial cells.²⁷ It has been utilized diagnostically as part of a prostate cancer imaging method (ProstaScint®, Cytogen Corp., Princeton, NJ), which uses a monoclonal antibody specific for PSMA (7E11.C5.3).²⁸ Enhanced expression of PSMA was detected in hormone refractory prostatic carcinoma.²⁹ In addition, levels of PSMA are elevated in the sera of hormone refractory advanced prostate cancer patients.³⁰ Our immunotherapy protocols use PSMA as target antigen for T cell attack in vivo.

T cell recognition requires binding of the TCR molecules to antigen-derived

peptides and MHC proteins. Previous studies have identified specific sequence motifs of MHC class I-binding peptides, making it possible to predict potential T cell epitopes from proteins of known amino acid sequences.³¹ Using these specific motifs, we identified the PSMA peptides with a high affinity for an MHC class I protein (HLA-A0201) expressed by a large fraction of the population to be used as antigens in our phase I and II clinical trials.^{32,33}

Phase I Clinical Trial

The phase I study,³⁴ conducted at Northwest Hospital in Seattle in December 1995, examined the safety of administering HLA-A0201-specific PSMA peptides (PSM-P1 and PSM-P2), autologous DC, and PSM-P1 and -P2 pulsed autologous DC to 51 patients with advanced hormone refractory prostate cancer. The majority of these patients (39/51) were classified as stage D₂ (T4N1-3M1a-c). Many of them were anemic and had undergone

various treatments that resulted in impaired immune competency. Fewer than 25% of these patients were considered fully immunocompetent at the start of the study as assessed by delayed-type hypersensitivity skin tests.

At the completion of four infusion cycles, the maximum tolerated dose had not yet been achieved. Neither significant acute nor chronic toxicity was observed at all doses of test substances, except for mild to moderate infusion-related hypotension. In addition, no significant increase in serum TNF α or interferon γ (IFN γ) was noted during the course of the study.³⁴

Patients were monitored for cellular immune modulation to the appropriate PSMA peptides (PSM-P1 or -P2). An enhanced cellular response was observed within the HLA-A2 positive subjects who were infused with DC pulsed with PSM-P1 or -P2.³⁴ Patient clinical responses were analyzed based on National Prostate Cancer Project (NPCP) criteria plus measurement of PSA levels. Seven patients exhibited partial responses.^{34,35} Average PSA levels increased in the non-responder group, both among those with low (0-19) and high (>19) pre-infusion values, while a decrease was observed in the seven partial responders (Fig. 2). All patients who were on hormone therapy before the trial continued their treatments during the trial. Patients who stopped anti-androgen therapy before the trial were not eligible to enter the study until three months had elapsed and the PSA measurement was at an acceptable level.

Phase II Clinical Trial

In January 1997, we started a phase II study involving 107 patients, 66 of whom had hormone refractory, clinically progressive prostate cancer (group A), and 41 of whom had locally recurrent prostate cancer (group B). Half of group A patients (33/66), participants in the phase I study, requested enrollment in the phase II trial. We anticipate that the results of

this phase II trial will provide information on the efficacy of our DC-based prostate cancer vaccine in both disseminated and localized prostate cancer.

As in the phase I study, group A subjects continued any hormone therapy that had been initiated before the trial.

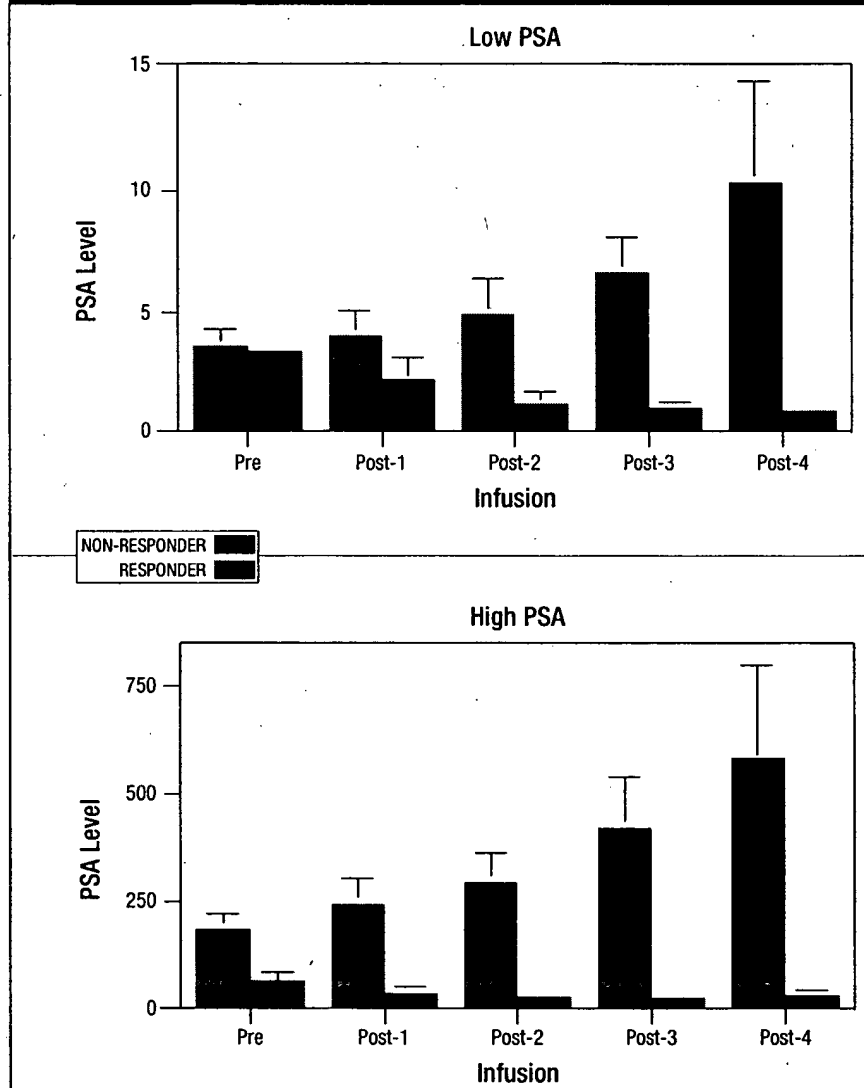
All phase II participants received a total of six infusions of autologous DC pulsed with a PSM-P1 and -P2 cocktail at six-week intervals. A subset of the participants in each group also received subcutaneous injection of GM-CSF as a systemic adjuvant with each DC infusion (Table 1). GM-CSF is a potent enhancer of differentiation for various hematopoietic cells, including DC.¹⁶ Moreover, administration of systemic GM-CSF promotes the outgrowth and antigen-presenting capabilities of DC.

All study participants were followed before, during, and after treatment with periodic measurements of PSA, free PSA, PSMA, complete blood counts, CHEM-22, and bone alkaline phosphatase, as well as with chest x-ray, bone scans, and ProstaScint scans. All testing was conducted on an outpatient basis at Northwest Hospital. Patients were also evaluated every infusion cycle by one of the study physicians. After the conclusion of the scheduled infusions and follow-up observations, clinical status was evaluated according to NPCP criteria and 50% reduction of PSA levels.

RESULTS

Thirty-three patients with metastatic prostate cancer in group A-I who had also participated in the phase I study (Table 2) were the first to complete the phase II study. Nine of the 33 subjects (27.3%) were identified as partial responders;³⁶ 11 patients (33.3%) exhibited no significant change during the phase II trial; and 13 patients experienced disease progression. Seven patients died during the study. Among the partial responders, four were also responders in the phase I study, with a total response period of over

Figure 2
PSA Levels Displayed According to Responder and
Non-Responder Categories



Each group is divided into low (initial PSA values 0-19) and high PSA (initial PSA values >19) categories. Normal ranges for PSA are 0-4 ng/ml. Mean and standard error of the mean (SEM) values were obtained from patient sera drawn pre-infusion and seven days post-infusion. There were 24 patients in the non-responder and two patients in the responder low PSA groups. There were 20 patients in the non-responder and five patients in the responder high PSA groups. In the latter, the mean PSA pre-infusion level for the non-responder group was 175.0 ng/ml, and at the end of four infusions, it was 583.4 ng/ml. For the responder group, the mean PSA values were 60.0 ng/ml at the pre-infusion level and 24.4 ng/ml at completion. In both groups, significant differences in pre- versus post-infusion values (p value < 0.05) were observed. Adapted from Tjoa et al,³⁵ with permission.

Table 1
The Phase II Clinical Trial: Treatment Groups

Group	Patient Category	Administration of GM-CSF (SC)	No. of Patients
A-I	Patients with metastatic prostate cancer: Phase I study participants who had requested enrollment in this study. Total: 33 patients	no	16
		yes	17
A-II	Patients with metastatic prostate cancer: Newly accrued patients. Total: 33 patients	no	20
		yes	13
B	Patients who had prostatectomy, brachytherapy, external radiotherapy, combined treatments, or hormones alone and had two successive PSA levels of 1.5 ng/ml or more. Total: 41 patients	no	27
		yes	14

370 days. Five additional responders in this phase II study had been non-responders in the phase I study. Their average partial response period was 196 days (Fig. 3). The total duration of this phase I/II study was 613 days. Twelve of 19 subjects (63%) with hormone refractory metastatic (D₂) prostate cancer survived for more than 600 days. We believe this is a significant observation because comparable patients with hormone refractory metastatic prostate cancer historically survive a median of only 6 months.³⁷

All scheduled infusions were completed by September 1998. A total of 95 evaluable subjects were assessed for response to treatment using NPCP criteria and 50% reduction in PSA levels. The NPCP response criteria included comparisons of pre- versus post-study bone scans and ProstaScint scans, which allowed us to evaluate both bony and nodal disease status.

Table 3 summarizes the clinical status of patients in groups A-II and B. In

group A-II, 32% of subjects experienced disease regression: Two patients (8%) were complete responders and six patients (24%) were partial responders. One patient exhibited no significant change, while 16 patients (64%) showed disease progression.

Ten group B participants (27%) were partial responders and one subject (3%) was a complete responder. Eight patients (22%) showed no significant change, and 18 patients (49%) showed disease progression.

IMMUNE RESPONSE ASSAYS

Several assays are being utilized to characterize the immune response to infused, peptide-pulsed DC. The frequency of antigen specific cells is expected to be quite low, on the order of 1 in 100,000 to 1 in 1,000,000 T lymphocytes, prior to immunization. Following infusion of peptide-pulsed DC, the frequency of antigen specific T cells may increase tenfold. De-

Table 2
Clinical Status of Phase II Subjects
(Based on NPCP Criteria, Plus 50% Reduction in PSA Levels)

Clinical Status	No. of Patients (%)	HLA-A2+(-)	D ₀	Stage D ₁	D ₂
Progression	13* (39.3%)	12 (1)	2	1	10
No change	11 (33.3%)	5 (6)	5	2	4
Partial response	9** (27.3%)	6 (3)	3	1	5
Total	33 (100%)	23 (10)	10	4	19

* Six patients from this group are deceased
 ** One patient from this group is deceased
 Adapted from Tjoa et al,³⁶ with permission.

TNM stages: T4NoMo T4N1-3MoT4N1-3M1a-c

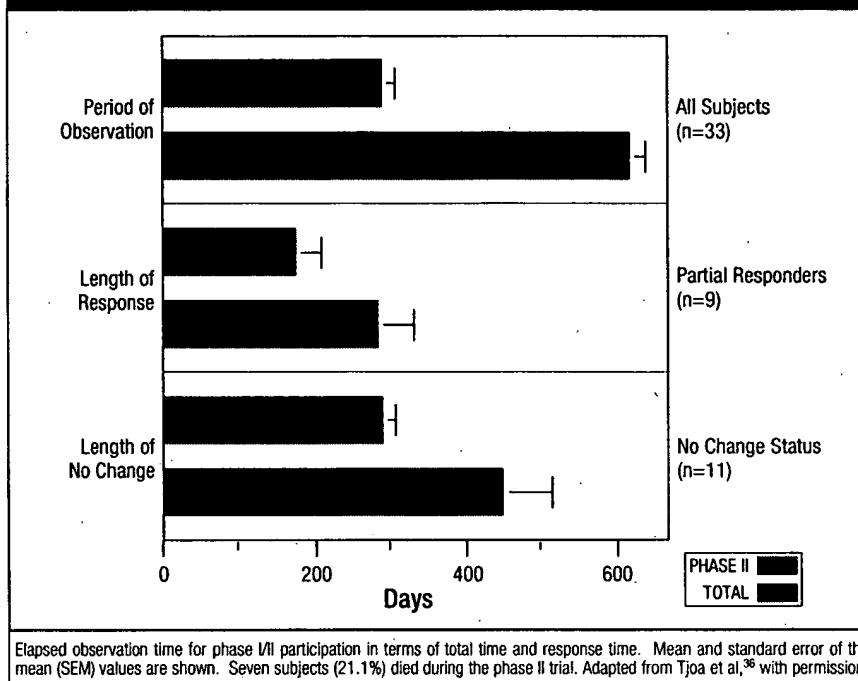
tection and quantitation of these low frequency cells requires very sensitive assays of T cell activity. The number of peptide specific T cells is quantitated in an ELISPOT assay based upon their secretion of IFN γ following in vitro stimulation with peptides.^{38,39} The amount of IFN γ secreted after stimulation with peptides is also measured, as is secretion of IL-10. Secretion of IFN γ indicates development of type 1 T cells, which are thought to represent the type of immune response required for anti-tumor effects. IL-10 is considered a regulatory cytokine produced by type 2 T cells. IL-10 has been shown to suppress antigen presentation by DC and to inhibit secretion of cytokines by type 1 T cells. Therefore, IL-10 secretion is monitored to detect immune responses that might inhibit anti-tumor effects. Cytokine secretion assays are performed on primary cultures of peripheral blood lymphocytes directly following isolation from peripheral blood and also after several rounds of in vitro stimulation. Repeated in vitro stimulation will increase the number of antigen

Table 3
Clinical Responses of Study
Participants in Groups A-II and B*

Group	Response	Total	%
A-II	CR	2	8
	PR	6	24
	NC	1	4
	P	16	64
	Total	25	
B	CR	1	3
	PR	10	27
	NC	8	22
	P	18	49
	Total	37	

* As of 9/17/98. CR=complete response; PR=partial response; NC=no change; and P=disease progression

Figure 3
Elapsed Observation Time for Phase I/II Participation



reactive cells to the level where they can be more readily detected.

Using these assays, responses to PSMA peptides were measured in patients participating in this clinical trial. In most patients analyzed, IFN γ production is detected and the response to peptides is transient. Two examples of this type of reactivity, measured by the ELISPOT assay, are depicted in Figure 4. T cells reactive to PSM-P2 were detected in the peripheral blood of patient 55 (Panel A) after two infusions of DC, but no reactivity was detected at later time points. This patient also experienced a decrease in serum PSA during the time that peptide reactive cells were detected in the blood. Similar data collected on patient 61 (Panel B) show a response to both PSMA peptides after two infusions of DC that

declines thereafter. Similar patterns are observed when total IFN γ secretion is measured. Secretion of IL-10 has also been observed in a minority of patients by two different assay systems.

Primary cultures of lymphocytes from patients 46 and 54 produce IL-10 when stimulated with PSMA peptides (Fig. 5). In both patients, this reactivity appeared to peak after four infusions and then decrease. As might be expected, given the role of IL-10 in the immune response, patient 54 has experienced disease progression. In the case of patient 46, serum PSA decreased during the period of DC infusions but rose after the final DC infusion. In testing lymphocyte cultures after three rounds of in vitro stimulation, two other patients have been identified as producing IL-10 in response to

Figure 4
Detection of Peptide-Reactive Peripheral Blood Mononuclear Cells

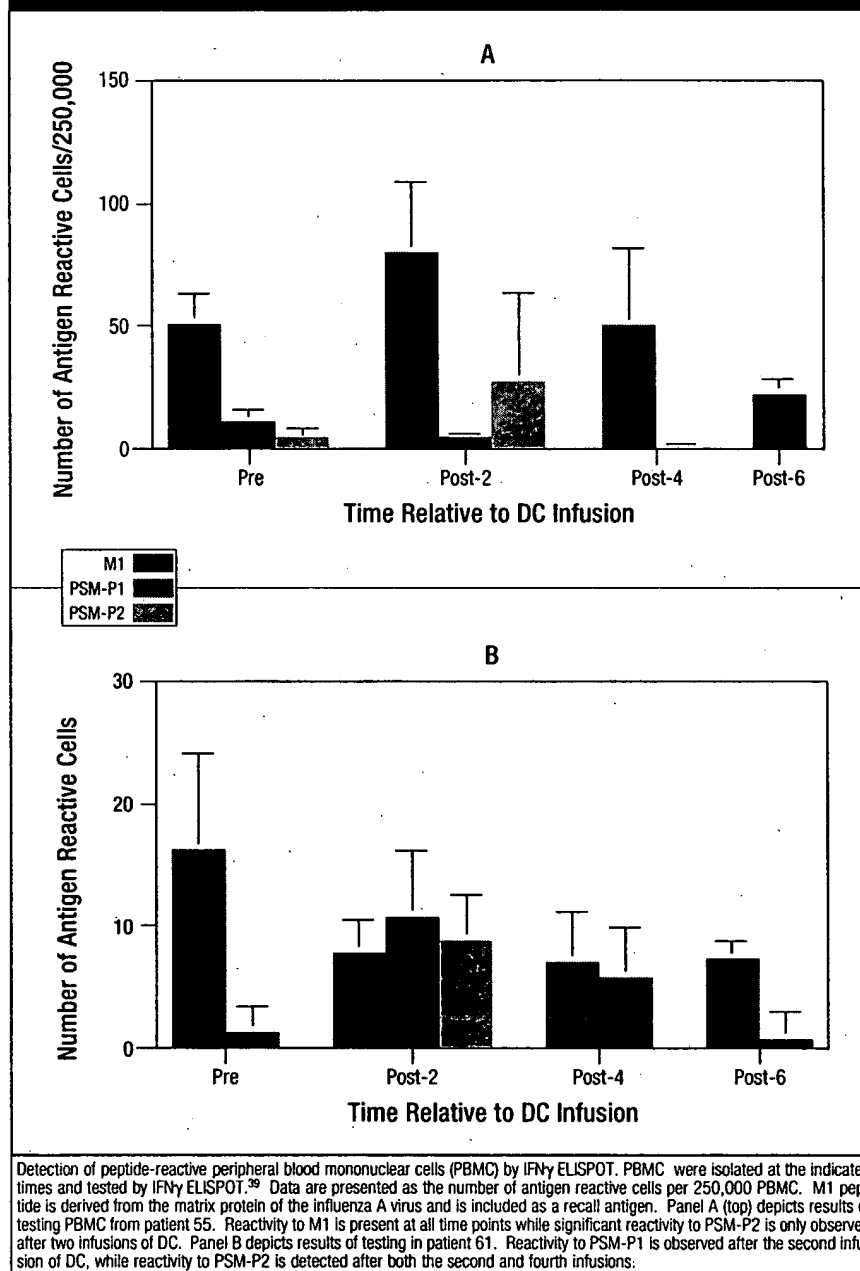
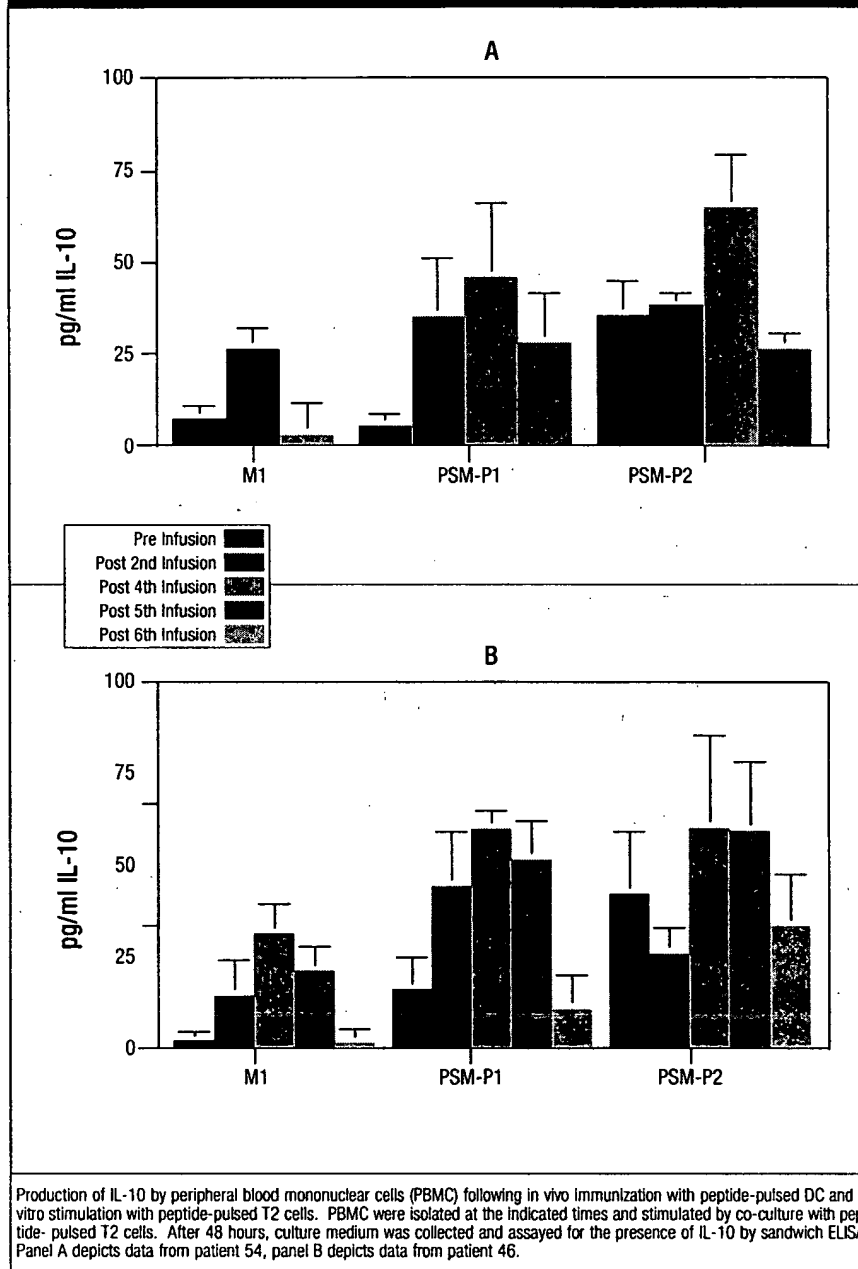



Figure 5
Production of IL-10



peptide stimulation. As more data are collected, the possible significance of these observations will be clarified.

Future Directions in Prostate Cancer Vaccine Development

The current studies demonstrate the potential of autologous DC as a vehicle to deliver specific target antigen, a crucial issue in prostate cancer vaccine develop-

ment. In our future clinical trials, we hope to expand the antigen repertoire of our vaccine study from two PSMA peptides (each of which represents nine amino acids) to a recombinant PSMA protein consisting of the native sequence without the transmembrane domain. This change will multiply the number of targets for T cell attack, and thus produce a potentially more effective vaccine. 

References

1. Landis SH, Murray T, Bolden S, Wingo PA: Cancer statistics, 1999. *CA Cancer J Clin* 1999;49:8-31.
2. Jones GW, Mettlin C, Murphy GP, et al: Patterns of care for carcinoma of the prostate gland: Results of a national survey of 1984 and 1990. *J Am Coll Surg* 1995;180:545-554.
3. Krikorian JG, Portlock CS, Cooney P, Rosenberg SA: Spontaneous regression of non Hodgkin's lymphoma: A report of nine cases. *Cancer* 1980;46:2093-2099.
4. Ralfkiaer E, Hou-Jensen K, Gatter KC, et al: Immunohistological analysis of the lymphoid infiltrate in cutaneous malignant melanomas. *Virchows Arch* 1987;410:355-361.
5. Ruiter DJ, Bhan AK, Harrist TJ, et al: Major histocompatibility antigens and mononuclear inflammatory infiltrate in benign nevocytic proliferations and malignant melanoma. *J Immunol* 1982;129:2808-2815.
6. Marrack P, Kappler J: The T cell receptor. *Science* 1987;238:1073-1079.
7. Jenkins MK, Johnson JG: Molecules involved in T-cell costimulation. *Curr Opin Immunol* 1993;5:361-367.
8. Weiss A, Littman D: Signal transduction by lymphocyte antigen receptors. *Cell* 1994;76:263-274.
9. Abbas AK, Lichtman AH, Pober JS: Cellular and Molecular Immunology, ed. 2. Philadelphia, WB Saunders, 1994.
10. Bear HD, Hamad GG, Kostuchenko PJ: Biologic therapy of melanoma with cytokines and lymphocytes. *Semin Surg Oncol* 1996;12:436-445.
11. Morgan D, Ruscetti FW, Gallo R: Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* 1976;193:1007-1008.
12. Rosenberg SA, Lotze MT, Yang JC, et al: Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* 1989;210:474-485.
13. Sedlacek H: Vaccination for treatment of tumors: A critical comment. *Crit Rev Oncogen* 1994;5:555-587.
14. Pardoll D: New strategies for active immunotherapy with genetically engineered tumor cells. *Curr Opin Immunol* 1992;4:619-623.
15. Urban JL, Schreiber H: Tumor antigens. *Annu Rev Immunol* 1992;10:617-644.
16. Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991;9:271-296.
17. Austyn JM: New insights into the mobilization and phagocytic activity of dendritic cells. *J Exp Med* 1996;183:1287-1292.
18. Knight SC, Farrant J, Bryant A, et al: Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. *Immunology* 1985;57:595-603.
19. Bernhard H, Disis ML, Heimfeld S, et al: Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. *Cancer Res* 1995;55:1099-1104.
20. Romani N, Gruner S, Brang D, et al: Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994;180:83-93.
21. Bender A, Sapp M, Schuler G, et al: Improved methods for the generation of dendritic cells from non-proliferating progenitors in human blood. *J Immunol Methods* 1996;196:121-135.
22. Tjoa B, Erickson S, Barren R 3rd, et al: In vitro propagated dendritic cells from prostate cancer patients as a component of prostate cancer immunotherapy. *Prostate* 1995;27:63-69.
23. Hsu FJ, Benike C, Fagnoni F, et al: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 1996;2:52-58.
24. Mukherji B, Chakraborty NG, Yamasaki S, et al: Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci U S A* 1995;92:8078-8082.
25. Nestle FO, Alijagic S, Gilliet M, et al: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328-332.
26. Wang MC, Valenzuela LA, Murphy GP, Chu TM: Purification of a human prostate specific antigen. *Investig Urol* 1979;17:159-163.
27. Israeli RS, Powell CT, Fair WR, Heston WD: Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res* 1993;53:227-230.
28. Horoszewicz JS, Kawinski E, Murphy GP: Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer

- patients. *Anticancer Res* 1987;7:927-935.
29. Kawakami M, Nakayama J: Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization. *Cancer Res* 1997;15:2321-2324.
30. Murphy GP, Ragde H, Kenny G, et al: Comparison of prostate specific membrane antigen, and prostate specific antigen levels in prostatic cancer patients. *AntiCancer Res* 1995;15:1473-1479.
31. Engelhard VH: Structure of peptides associated with class I and class II MHC molecules. *Annu Rev Immunol* 1994;12:181-207.
32. Tjoa B, Boynton A, Kenny G, et al: Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* 1996;28:65-69.
33. Salgaller ML, Lodge PA, McLean JG, et al: Report of immune monitoring of prostate cancer patients undergoing T-cell therapy using dendritic cells pulsed with HLA-A2-specific peptides from prostate-specific membrane antigen (PSMA). *Prostate* 1998;35:144-151.
34. Murphy GP, Tjoa B, Ragde H, et al: Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate* 1996;29:371-380.
35. Tjoa BA, Erickson SJ, Bowes VA, et al: Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* 1997;32:272-278.
36. Tjoa BA, Simmons SJ, Bowes VA, et al: Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides. *Prostate* 1998;36:39-44.
37. Klein LA: Prostatic carcinoma. *N Engl J Med* 1979;300:824-833.
38. Herr W, Schneider J, Lohse AW, et al: Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J Immunol Methods* 1996;191:131-142.
39. Scheibenbogen C, Lee KH, Stevanovic S, et al: Analysis of the T cell response to tumor and viral peptide antigens by an IFNgamma-ELISPOT assay. *Int J Cancer* 1997;71:932-936.

Immunotherapy of Hormone-Refractory Prostate Cancer With Antigen-Loaded Dendritic Cells

By Eric J. Small, Paige Fratesi, David M. Reese, George Strang, Reiner Laus, Madhusudan V. Peshwa, and Frank H. Valone

Purpose: Provenge (Dendreon Corp, Seattle, WA) is an immunotherapy product consisting of autologous dendritic cells loaded ex vivo with a recombinant fusion protein consisting of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor. Sequential phase I and phase II trials were performed to determine the safety and efficacy of Provenge and to assess its capacity to break immune tolerance to the normal tissue antigen PAP.

Patients and Methods: All patients had hormone-refractory prostate cancer. Dendritic-cell precursors were harvested by leukapheresis in weeks 0, 4, 8, and 24, loaded ex vivo with antigen for 2 days, and then infused intravenously over 30 minutes. Phase I patients received increasing doses of Provenge, and phase II patients received all the Provenge that could be prepared from a leukapheresis product.

Results: Patients tolerated treatment well. Fever, the most common adverse event, occurred after 15 infusions (14.7%). All patients developed immune responses to the recombinant fusion protein used to prepare Provenge, and 38% developed immune responses to PAP. Three patients had a more than 50% decline in prostate-specific antigen (PSA) level, and another three patients had 25% to 49% decreases in PSA. The time to disease progression correlated with development of an immune response to PAP and with the dose of dendritic cells received.

Conclusion: Provenge is a novel immunotherapy agent that is safe and breaks tolerance to the tissue antigen PAP. Preliminary evidence for clinical efficacy warrants further exploration.

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PROSTATE CANCER IS the most common type of cancer and the second leading cause of death as a result of cancer in North American men.¹ Metastatic disease is initially treated with androgen deprivation, which achieves stabilization or regression of disease in more than 80% of patients.² However, despite androgen deprivation and secondary hormonal manipulations,³ all patients ultimately develop hormone-refractory prostate cancer (HRPC). The median survival for this group of patients is approximately 1 year. To date, no agent has been shown to prolong survival in HRPC patients,^{4,5} and prospectively validated palliative interventions are few. Novel therapeutic agents for the treatment of HRPC are urgently required.

Active immunotherapy of cancer seeks to induce tumor-specific immunity in the patient and is consequently dependent on a suitable target antigen and effective presentation

of that antigen to the patient's immune system. Antigen-presenting cells (APCs) are responsible for uptake, processing, and presentation of antigens to T cells of the immune system in the context of HLA class I and class II molecules. Dendritic cells are the most potent APCs and the only APCs that can prime an immune response by T cells that have not been exposed to the antigen previously.^{6,7}

Immunotherapy with dendritic cells loaded with specific tumor antigens ex vivo has been studied extensively in animals.⁸⁻¹¹ All of these studies found dendritic cells to be effective in treating or preventing tumors in experimental animals in an antigen-specific fashion. Several pilot clinical studies using dendritic cells to deliver antigen for immunotherapy of human malignancies have also shown promise and demonstrate that dendritic-cell therapy can elicit a beneficial immune response.¹²⁻¹⁸

In many of the preclinical and clinical studies described above, the antigen targets that have proven to be useful in cancer are tissue-specific proteins to which the immune system is normally tolerant. Preclinical studies in rats aimed at eliciting prostate-specific immunity demonstrated that dendritic cells loaded with an engineered antigen-cytokine fusion protein (PA2024) consisting of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce strong cellular immune responses in vivo to tissues and tumors that express PAP.¹⁹ Delay of tumor development and improved survival were observed in tumor prevention models. In contrast, dendritic cells pulsed with PAP alone elicited significantly weaker

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immune responses, indicating an important role for the GM-CSF portion of the fusion protein in antigen presentation. Dendritic cells are likewise essential for eliciting cellular immunity in this model, as injections of the PAP-GM-CSF fusion protein alone and injections of PAP in Freund's adjuvant elicited antibody responses but not cellular immune responses to PAP. Based on these preclinical observations, a dendritic-cell product (Provenge [Dendreon Corp, Seattle, WA], or APC8015) consisting of autologous dendritic cells loaded with the human PAP-GM-CSF fusion protein was developed, and clinical testing of Provenge was undertaken in HRPc patients in a phase I/II trial.

PATIENTS AND METHODS

Patients

Eligible patients had histologically confirmed adenocarcinoma of the prostate with evidence of disease progression despite androgen deprivation (and if applicable, antiandrogen withdrawal), serum testosterone less than 50 ng/mL, and an expected survival of at least 3 months. Patients with negative bone scan and computed tomography scan were eligible, provided there were at least three climbing prostate-specific antigen (PSA) values, at least 2 weeks apart from each other, and at least 1 month or more after antiandrogen withdrawal. Other eligibility requirements included an Eastern Cooperative Oncology Group performance status of 0 or 1, a serum PAP level ≥ 2 times the upper limits of normal, or positive staining for PAP by immunohistochemistry on any prior prostatic cancer specimen. Negative serologic tests for human immunodeficiency virus (HIV), human T-cell leukemia virus type 1, hepatitis B, and hepatitis C were required, as were adequate hematologic, renal, and hepatic function (WBC $\geq 2,000/\mu\text{L}$, ANC $\geq 1,000/\mu\text{L}$, platelets $\geq 100,000/\mu\text{L}$, hemoglobin > 9.0 g/dL, creatinine ≤ 2.0 mg/dL, total bilirubin \leq two times upper limit of normal, and ALT and AST five times upper limit of normal). Prior chemotherapy, investigational agents, megestrol acetate or other hormones, or saw palmetto, PC-SPES (Botanic Labs, Brea, CA), or other herbal preparations were allowed, provided they were discontinued at least 1 month before treatment and the patient had recovered adequately. Prior radiation therapy had to have been completed at least 1 month before treatment, and radiopharmaceuticals could not have been administered within 2 months of treatment. Patients who required systemic corticosteroids for any indication were not eligible.

Treatment/Clinical End Points

Patients without prior orchiectomy continued on gonadal suppression with a luteinizing hormone-releasing hormone agonist. Patients were treated with a fixed dose of Provenge on weeks 0, 4, and 8. A fourth infusion was administered on week 24 to patients whose disease was stable or improving. During the phase I portion of the study, the dose of Provenge was escalated for cohorts of three patients based on treatment-related toxicity. The planned dose levels were 0.2×10^9 , 0.6×10^9 , 1.2×10^9 , and 2.0×10^9 nucleated cells/ m^2 (the upper limit of testing was defined by the anticipated maximum manufacturable dose of Provenge). No intrapatient dose escalation was undertaken. Sixteen additional patients were scheduled to receive the maximum-tolerated dose of Provenge or the maximum dose of Provenge that could be prepared. Patients no. 2 through 6 also received one or two

doses of APC8017 (keyhole limpet hemocyanin [KLH]-loaded dendritic cells) as an internal positive control. The leukapheresis products for these patients were split into two aliquots for preparation of both Provenge and APC8017. The dose of APC8017 was all the cells that could be prepared from the split leukapheresis unit. Patients were observed until objective disease progression or 1 year, whichever was first. Serum PSA levels were measured every 4 weeks until disease progression. Time to progression was defined as the time from the day of registration until the day objective disease progression was documented. Patients who elected to come off study without objective disease progression (eg, for increasing PSA) were considered to have disease progression at the time of study withdrawal.

Preparation and Administration of Provenge

Provenge (APC8015) was prepared fresh for each treatment course. Dendritic-cell precursors were harvested from the peripheral blood by a standard 1.5 to 2.0 blood volume mononuclear cell leukapheresis. Mobilization with a colony-stimulating factor was not required. The leukapheresis products were prepared at a local blood bank and transported within 1 hour to the local Dendreon cell processing facility in Mountain View, CA. Dendritic-cell precursors were collected by two sequential buoyant density centrifugation steps by a modification of the method of Hsu et al.^{12,17,20} Briefly, the leukapheresis product is layered over a buoyant density solution (specific gravity = 1.077 g/mL) and centrifuged at 1,000 g for 20 minutes to deplete erythrocytes and granulocytes. The interface cells are collected, washed, layered over a second buoyant density solution (specific gravity = 1.065 g/mL), and centrifuged at 805 g for 30 minutes to deplete platelets and low-density monocytes and lymphocytes. The cell pellet, which contains dendritic-cell precursors, is washed and incubated in AIM media with 10 $\mu\text{g}/\text{mL}$ of the appropriate target antigen (PA2024 to prepare Provenge or KLH to prepare APC8017). The culture media did not contain serum or exogenous cytokines. After incubation for 40 hours at 37°C in 5% CO₂ atmosphere, the cells were washed and formulated at the desired clinical dose in 250 mL of lactated Ringers' solution. Criteria for releasing the products for infusion included the following: (1) in-process sterility tests with no growth at 40 hours; (2) endotoxin less than 1.4 EU/mL; (3) CD54 expression greater than 3 SD above T = 0 value; (4) cell viability greater than 72%; and (5) phenotype consistent with the values listed in Table 2. Phenotype was determined by flow cytometry using monoclonal antibodies to CD4, CD8, CD54, CD56, CD66b, and CD86 (Becton Dickinson, San Jose, CA; Coulter, Miami, FL). Additional tests, the results of which were available after infusion, were final product sterility, mycoplasma, and allogeneic mixed-lymphocyte reactivity (alloMLR).

The final Provenge products were transported to the outpatient infusion center at the University of California, San Francisco, Comprehensive Cancer Center at 4°C and infused into the patients within 8 hours of formulation. Provenge and, when appropriate, APC8017 were infused separately, each over 30 minutes, beginning with Provenge. Patients were not routinely premedicated before the infusion. They were observed for 30 minutes after infusion and then discharged to home.

PA2024, the target antigen used to prepare Provenge, is a fusion protein consisting of full-length human PAP and full length human GM-CSF. The fusion protein was cloned in a baculovirus system and expressed in Sf21 insect cells adapted to grow in serum-free media. PA2024 was purified by three sequential column chromatography steps to more than 95% homogeneity. Both protein components retained appropriate biologic activity, as demonstrated by enzymatic activity for PAP and growth promotion activity for GM-CSF.

Immune Function Testing

AlloMLR. Samples of each lot of Provenge and APC8017 were tested for their ability to stimulate alloMLR using a standard lymphocyte proliferation assay.²¹ A pool of responder T cells was prepared using buffy coats from normal donors. The cells were purified to 93% CD3+ cells, using a T-cell column from R&D Technologies (Minneapolis, MN), and frozen in aliquots. The pooled T cells did not proliferate in the absence of allogeneic APCs, indicating the T cells were not contaminated by APCs. The purified T cells (100,000/well) were cultured with serial dilutions of irradiated product starting at 400,000 cells/well.

T-cell proliferation. Standard T-cell proliferation assays were performed using peripheral-blood lymphocytes isolated from the blood of each patient, obtained at the beginning of each leukapheresis.²¹ These samples (10⁵ cells/well) were incubated with increasing concentrations of antigen (PA2024 or KLH) for 5 days at 37°C, at which point each well was pulsed with ³H thymidine. Twenty-four hours later, cultures were harvested and mean radioactivity incorporated into proliferating cells was determined. Data are reported as either counts per minute (CPM) or as the stimulation index (SI) (SI = [mean CPM antigen]/mean CPM control).

Allogeneic and Autologous T-Cell Stimulation Activity Before and After Ex Vivo Culture

T cells were purified from peripheral-blood mononuclear cells of pooled allogeneic donors, and autologous leukapheresis product using CD3 T-cell enrichment columns and were cryopreserved for use as responder cells in functional assays. Stimulator cells consisted of cells obtained from either precursor dendritic-cell product (preculture) or APC8015/Provenge (postculture). Responder cells (5 × 10⁴ cells/well) were mixed with various numbers of irradiated (3,000 rads) stimulator cells and were cultured for 5 days in AIM-V medium supplemented with 5% pooled human AB serum. Subsequently, tritiated thymidine was added overnight, and its incorporation was determined to generate T-cell proliferation dose response curves to assess the allogeneic and autologous T-cell stimulatory capacity of the stimulator cells.

ELISPOT

ELISPOT assays for interferon-gamma (IFN γ) were performed using Millipore HA plates containing cellulose ester membranes coated with murine antgamma IFN.²² A reader blinded to the identities of the samples determined the number of spots (cells secreting IFN γ), and the frequency of responding cells was calculated by dividing the number of spots in triplicate wells by the number of cells in the wells.

Enzyme-Linked Immunoassay (ELISA) for Antibodies and Cytokines

ELISAs for antibodies to the immunogen were performed, as described,²³ using serum collected before treatment and every 4 weeks after treatment. To evaluate cytokine secretion by T cells, supernatants from proliferation assays were tested for the presence of IFN γ and interleukin (IL)-4 using commercial ELISA kits (Endogen, Woburn, MA).

Statistical Design

A standard modified Fibonacci phase I design was used, with three patients tested at each dose level. In addition to six patients treated at the maximum dose in the phase I trial, an additional 16 patients were

to be treated at this dose in the phase II trial. If no responses were seen in these patients, then it would be concluded that the probability that the true response rate was $\geq 15\%$ was less than 0.05.

T-cell proliferation data are not normally distributed. To normalize the data for statistical analyses, the data were expressed as the proliferation quotient (PQ) [PQ = (log CPM antigen) - (log CPM control)]. Different patient groups were compared by paired or non-paired *t* tests as appropriate.

RESULTS

Patients

A total of 31 patients were treated. Twelve men were treated in the phase I portion, with six patients treated at the maximum dose of Provenge. Nineteen men were enrolled onto the phase II trial at the maximum dose (representing an over-accrual of three patients to the phase II portion to account for potentially unassessable patients). Patient characteristics are listed in Table 1. Median age was 69 years (range, 48 to 83 years). Median Eastern Cooperative Oncology Group performance status was 0 (range, 0 to 1).

Median PSA was 41.3 ng/mL (range, 3.4 to 1,007 ng/mL). In the phase I component, all 12 patients had metastatic disease, and the median PSA was 209 ng/mL (range, 26.3 to 1,007 ng/mL). The patients in the phase I trial were more heavily pretreated. All had undergone androgen deprivation with combined androgen blockade, followed by antiandrogen withdrawal. Eleven (92%) of 12 patients had received a second-line hormonal manipulation, such as ketoconazole, and eight patients (66%) had also received chemotherapy, suramin, or some other investigational agent. By contrast, the patients in the phase II portion had less extensive disease. None of these patients had metastases identified on bone scan or computed tomography. An increasing PSA was the only evidence of disease progression, and the median PSA level was much lower (14.5 ng/mL; range, 3.4 to 216 ng/mL). All 19 phase II patients had undergone combined androgen deprivation followed by antiandrogen withdrawal. Twelve (63.2%) had received a second-line hormone, and only one had received prior therapy with an investigational agent (hydrazine sulfate).

Preparation of PAP-Loaded Dendritic Cells (Provenge)

One hundred two lots of Provenge were prepared for the 31 patients. Leukapheresis was performed with peripheral venous access for all but two patients who required placement of central venous catheters. Dendritic-cell precursors matured during culture, as evidenced by upregulation of costimulatory molecules and increased potency in allogeneic and autologous T-cell stimulation activity as shown in Figs 1 and 2. We correlated the expression of costimulatory molecules with potency in the alloMLR in 81 consecutive

Table 1. Patient Characteristics

	Phase I (no. of patients)	Phase II (no. of patients)	Total (no. of patients)
Demographics			
Patients enrolled	12	19	31
Age, years			
Median	67.5	72	69
Range	48-79	50-83	48-83
PSA, ng/mL			
Median	209	14.5	41.3
Range	26.3-1,007	3.4-216	3.4-1,007
Performance status			
Median	0	0	0
Range	0-1	0-1	0-1
Primary therapy			
Prostatectomy	6	4	10
Radiation	2	7	9
Cryosurgery	1	0	1
Extent of disease			
Bone disease only	10	0	10
Bone and soft tissue	2	0	2
PSA only	0	19	19
Prior systemic therapy			
Androgen deprivation	12	19	31
Second-line hormones	11	12	23
Chemotherapy	8	0	8
Other	3	1	4

lots of Provenge and found that potency correlated most strongly with expression of CD54 ($P < .0001$; two-tailed t test with $\alpha = 0.05$). Cell sorting experiments revealed that all antigen-presenting activity resided in the CD54+ population (data not shown). Based on these observations, we selected CD54 expression as a marker of dendritic cells and product potency. Although CD54 is not a specific marker for dendritic cells, because of its association with potency in the alloMLR and the cell sorting experiments, we have used it as an important characterizing marker of the cell product used, and for the purposes of this article, refer to CD54(+) cells as dendritic cells. Table 2 lists the characteristics of the Provenge preparations. The median number of nucleated cells was 2.1×10^9 cells. Of these cells, 11.2% were CD54(+), resulting in a median number of dendritic cells infused of 123×10^6 cells. Although there was a wide range in the number of dendritic cells infused, this was because of patient-to-patient variability. As expected, phase I patients received fewer dendritic cells because of the planned dose escalation. Analysis of lineage markers revealed expression of CD3 (T cells) in 62.3%, CD19 (B cells) in 7.2%, CD14 (monocytic cells) in 11.7%, and CD56 (natural killer cells) in 14.4% of the nucleated cells in Provenge. Thus, Provenge consists of CD54(+) cells that constitute the APC population and other immunologically active cells, including T cells and B cells.

Adverse Events

Overall treatment was well tolerated. Most patients had no treatment-related adverse events. Other than minor discomfort, there were no adverse events associated with leukapheresis.

Fifteen infusions (14.7%) were associated with febrile reactions that developed within 2 hours. Two febrile reactions were scored as grade 3 using National Cancer Institute common toxicity criteria, and 13 were grade 1 or 2. Similarly, mild myalgias (grade 1) occurred 1 or 2 days after Provenge infusions in two patients, and mild fatigue occurred in one patient. Five patients experienced mild urinary complaints, including obstructive voiding symptoms, incontinence, urgency, and nocturia. There was no treatment-related hematologic, hepatic, or renal toxicity.

Stimulation of Antigen-Specific Immune Responses

The patients' T-cell and B-cell (antibody) responses to the PAP-GM-CSF construct PA2024 were measured before treatment and every 4 weeks thereafter. The 31 patients had little or no pre-existing T-cell proliferation responses to PA2024, whereas 100% developed T-cell proliferation responses after infusion of Provenge. Figure 3 presents the results of lymphocyte proliferation assays at 0 and 4 weeks for one patient, who received both PAP and KLH-loaded

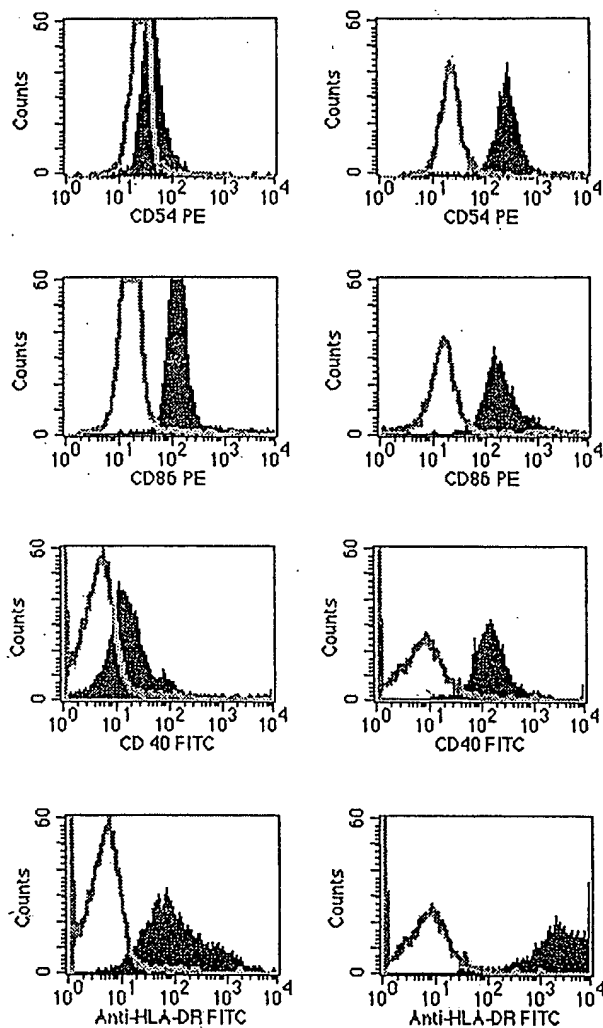


Fig 1. Expression of the costimulatory molecules CD54, CD86, and CD40 and of HLA-DR was determined at the beginning (left panel) and after 40 hours of culture (right panel) by flow cytometry. Culture ex vivo was associated with upregulation of costimulatory molecule expression.

dendritic cells. Figure 4 presents all T-cell proliferation data for the phase I patients through week 12. The T-cell proliferation responses to the fusion protein were maximal after either two or three infusions of Provenge. For the cohort of 12 patients, the response was significantly higher at week 4 compared with week 0 ($P < .01$) and at week 8 compared with week 4 ($P < .05$), but not at week 12 compared with week 8. The dose of Provenge infused did not correlate with the magnitude of the T-cell proliferation response. Phase II patients had immune responses similar to the phase I patients (data not shown).

To exclude the possibility that Provenge stimulates T-cell responses nonspecifically, the patients' T-cell proliferation

responses to the recall antigen influenza and to the naive antigen KLH were measured before treatment and every 4 weeks thereafter. The 12 phase I patients' T-cell stimulation index to influenza did not change with treatment. The median stimulation index was 5.5 at week 0 and 4.7 at week 8 for the lowest in vitro antigen dose ($0.4 \mu\text{g/mL}$) and 9.2 at week 0 and 9.7 at week 8 for the highest dose ($50 \mu\text{g/mL}$). Five patients received KLH-loaded dendritic cells (APC8017). None had pre-existing T-cell proliferation responses to KLH and, as expected, all developed responses after treatment with APC8017.

By contrast, nine patients who did not receive APC8017 were tested for KLH immune responses after treatment with PAP-loaded dendritic cells (Provenge), and none developed a response to KLH. Thus, Provenge stimulated antigen-specific immune responses.

PA2024 consists of PAP fused to the targeting element GM-CSF. T-cell responses to each of these components were examined. No patient had pre-existing T-cell responses to PAP isolated from human seminal fluid; whereas after treatment with Provenge, 10 (38%) of 26 patients developed a T-cell response to PAP. Pre-existing T-cell proliferation responses to GM-CSF (Leukine; Immunex, Seattle, WA) were observed in 15 patients (57%), of whom three had been treated previously with GM-CSF on a different immunotherapy protocol. After treatment with Provenge, T-cell proliferation responses to GM-CSF were observed in an additional four patients for a total of 19 (70%) of 27 patients.

T cells can be separated into two distinct groups based on the type of cytokines the cells secrete. Th1 cells secrete $\text{IFN}\gamma$, whereas Th2 cells secrete IL-4 and IL-10. The patients' pretreatment T cells did not secrete either $\text{IFN}\gamma$ or IL-4 in response to PA2024, indicating a Th1-type response (data not shown). In addition, ELISPOT assays of cytokine secretion by single lymphocytes showed that the frequency of cells secreting $\text{IFN}\gamma$ in response to PA2024 increased from undetectable ($< 1/10^6$ cells) to $1/5763$ cells and $1/5181$ cells for the two patients who were studied.

Antibodies to PAP and GM-CSF were evaluated by specific ELISA on serum samples obtained at baseline and then every 4 weeks. None of the patients had pre-existing antibodies to PAP (isolated from human seminal fluid); whereas after treatment, 16 (52%) of 31 patients had antibodies. The median antibody titer was $1/240$ (range, $1/40$ to $1/5120$). Similar to the T-cell experience, 10 patients (33%) had pre-existing antibodies to GM-CSF, and after treatment, 25 (80.6%) of 31 patients had antibodies.

The baseline immune function of all patients was assessed by in vitro T-cell proliferation responses to the recall

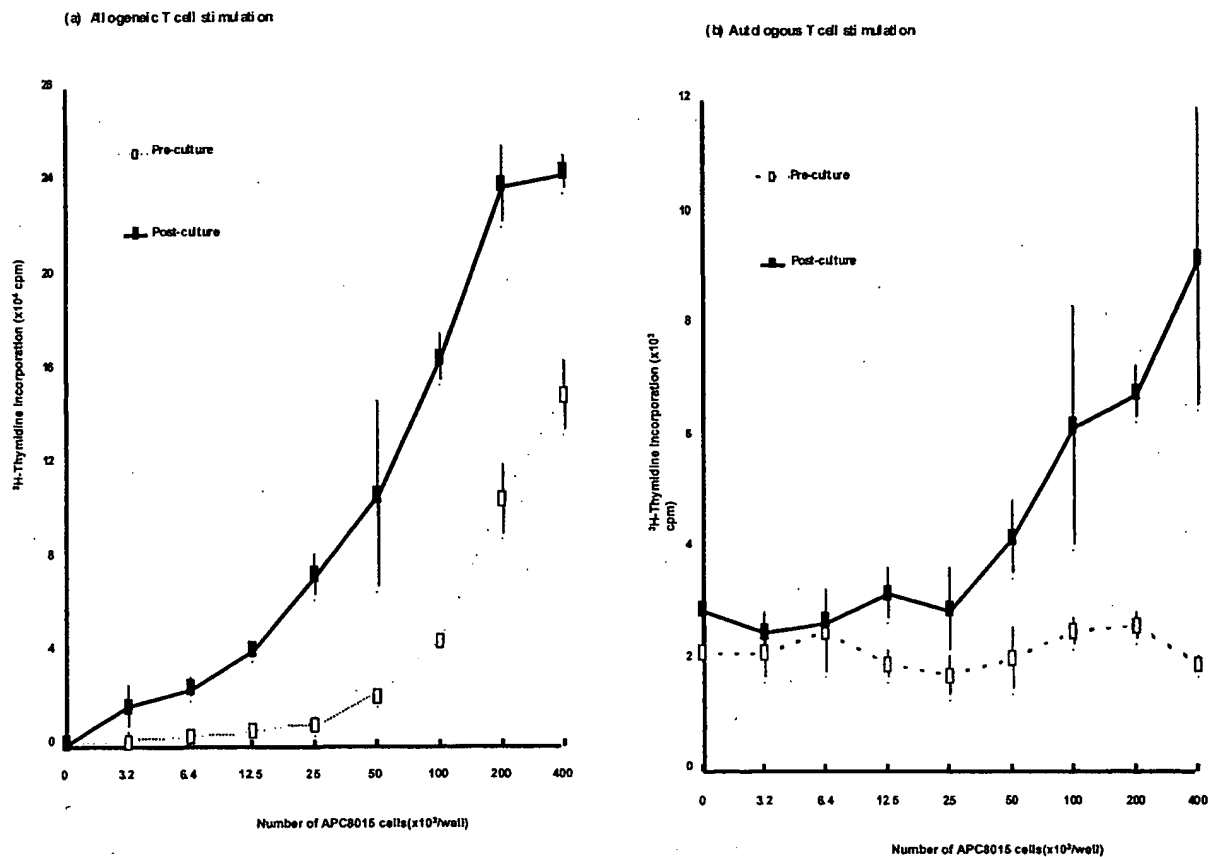


Fig 2. Forty-hour ex vivo culture is accompanied by an increase in (A) allogeneic and (B) autologous T-cell stimulation activity. T cells obtained from peripheral-blood mononuclear cells of allogeneic donors and autologous leukapheresis product were used as responder cells. Stimulator cells consisted of precursor dendritic cells before and after culture with prostate antigen PA2024.

antigen influenza. There was no difference in baseline immune response to influenza between patients who did or did not subsequently develop an immune response to PAP. Similarly, there was no difference in baseline immune responses to influenza between patients who received an average dose of more than 100×10^6 cells and those who received fewer cells.

Responses to Treatment

Three patients had a $\geq 50\%$ decrease in serum PSA, and three more patients had 25% to 49% decreases in PSA. No improvements in bone scans or soft tissue disease were observed. The median time to disease progression for the phase I patients was 12 weeks, and the median time to progression for the phase II patients was 29 weeks. Seven of the 19 phase II patients had not progressed by the end of the planned 1-year follow-up period.

The relationship between development of a T-cell or B-cell immune response to PAP (seminal fluid-derived) and

the time to disease progression was evaluated (Fig 5). The median time to disease progression was 34 weeks for patients who developed an immune response ($n = 20$) compared with 13 weeks for patients who did not ($n = 11$) ($P < .027$).

The relationship between the time to disease progression and the average dose of dendritic cells received by each patient was also examined. Inspection of the data revealed that all patients who experienced disease progression more than 24 weeks after registration received average cell doses above 100×10^6 cells/infusion. The median time to disease progression was 31.7 weeks for patients who received more than 100×10^6 cells/infusion compared with 12.1 weeks for patients who received fewer cells (Fig 6). The difference between the two groups was statistically significant ($P = .013$).

DISCUSSION

This phase I/II trial demonstrates that treatment of men with HRPc with Provenge induced specific immune re-

Table 2. Phenotype and Function of Provenge

	Phase I	Phase II	Total
No. of products	36	66	102
No. of nucleated cells, $\times 10^6$			
Median	1,308	2,376	2,172
Range	230-2,784	216-3,108	216-3,108
No. of CD54(+) cells, presumed dendritic cells, $\times 10^6$			
Median	30	278	123
Range	1.4-488	18.6-1,276	1.4-1,276
Phenotype markers, % of cells positive, mean \pm SD			
CD54, dendritic cells	4.6 \pm 5.8	14.8 \pm 12.3	11.2 \pm 11.5
CD3, T cells	69.3 \pm 15.8	58.5 \pm 15.5	62.3 \pm 16.4
CD19, B cells	8.1 \pm 5.9	6.7 \pm 2.8	7.2 \pm 4.2
CD14, monocytic cells	5.9 \pm 6.3	14.8 \pm 11.1	11.7 \pm 10.5
CD56, natural killer cells	14.1 \pm 7.8	14.6 \pm 6.7	14.4 \pm 7.1
AlloMLR EC50, $\times 10^4$ cells,* mean \pm SD	13.1 \pm 17.5	14.4 \pm 37.9	13.8 \pm 30.3

*The data are the number of cells ($\times 10^4$) that stimulate half-maximal proliferation of purified allogeneic T lymphocytes. The number of T cells used as responders was 100,000 per well.

sponses in all patients, with the response being apparent after a single treatment. Specificity of this therapy is suggested by the fact that treatment with Provenge did not increase the patients' response to the recall antigen influenza. In addition, none of the patients who received Provenge alone developed immune responses to the control antigen KLH. Cytokine production by T cells responding to the target antigen was analyzed by ELISA in some of the patients. The profile of cytokines produced indicates that the patients' T cells released IFN γ but not IL-4. These data suggest that the T-cell response was of the Th-1 type, which is thought to be important for host immunity to tumors. The

baseline immune function of all patients was assessed by in vitro T-cell proliferation responses to the recall antigen influenza. There was no difference in baseline immune response to influenza between patients who did or did not subsequently develop an immune response to PAP. Similarly, there was no difference in baseline immune responses to influenza between patients who received an average dose of more than 100×10^6 cells and those who received fewer cells.²⁴ ELISPOT assays in two patients confirmed the Th-1 cytokine profile and revealed substantial increases in T-cell precursor frequency.

The GM-CSF element in our prostate antigen is essential to in vitro antigen processing, but there are several reasons why we believe that GM-CSF does not otherwise contribute to Provenge's in vivo effects. First, the cells are washed extensively before infusion, and the quantity of residual GM-CSF is negligible. Secondly, most investigators use dendritic cells prepared in the presence of GM-CSF, and there is, to our knowledge, no evidence that the in vivo activity of those dendritic cells is caused by an adjuvant effect of GM-CSF. Thirdly, our preclinical studies compared infusions of dendritic cells pulsed with the fusion protein with injections of the fusion protein itself. Unlike the antigen-pulsed dendritic cells, the PAP-GM-CSF fusion protein did not elicit T-cell responses to PAP. Finally, we have performed a clinical trial that involved subcutaneous injections of the fusion protein and observed that the injections did not stimulate T-cell or antibody responses.²⁵

Several groups have reported pilot trials of antigen-loaded dendritic cells for solid and hematologic malignancies and for HIV infection.¹⁷ Hsu et al¹² treated four B-cell lymphoma patients with immunoglobulin idiotype-pulsed

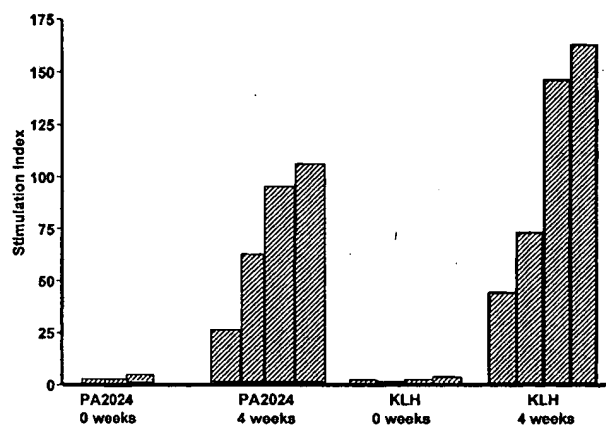
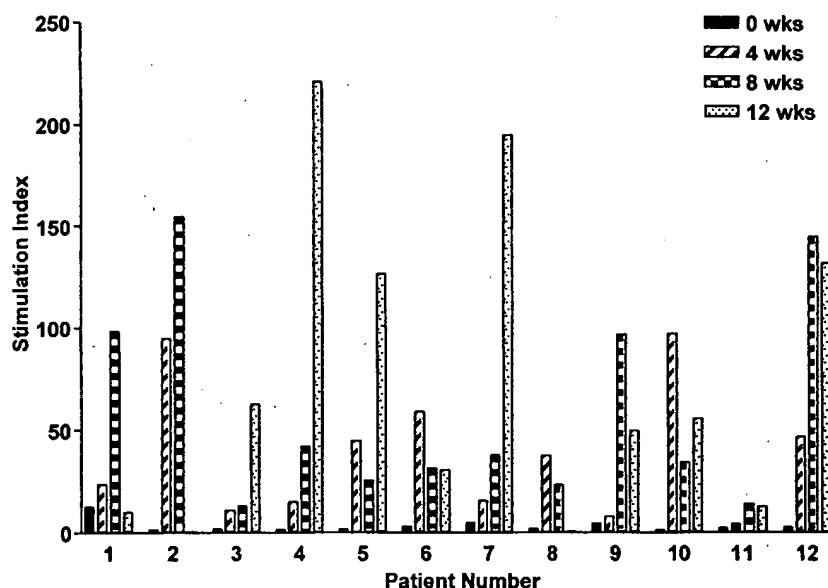


Fig 3. Mononuclear cells were isolated from patient no. 2 before infusion and 4 weeks after the first infusion of dendritic cells pulsed with prostate antigen PA2024 or KLH. Each group of four columns reflects lymphocyte proliferation assays undertaken at the following four concentrations of antigen: 0.4 μ g/mL (column 1), 2 μ g/mL (column 2), 10 μ g/mL (column 3), and 50 μ g/mL (column 4). Treatment with antigen-pulsed dendritic cells induced T-cell responses to PA2024 and KLH.

Fig 4. T-cell proliferative responses to PA2024 (10 μ g/mL) for all 12 phase I patients. Patients received infusions of Provenge on weeks 0, 4, and 8.



dendritic cells and observed two complete remissions. Treatment with idiotypic-pulsed dendritic cells has resulted in disease regression in 25% of low-tumor burden myeloma patients¹³ and in disease stabilization of high-tumor burden myeloma patients.¹⁴ Peptide-pulsed or tumor-lysate pulsed dendritic cells have yielded clinical regressions in patients with advanced melanoma.¹⁵ Similarly, fusion of autologous renal cell carcinoma cells with allogeneic dendritic cells has resulted in complete regression of tumor in some renal cell carcinoma patients.²⁶ Carcinoembryonic antigen peptide-pulsed dendritic cells stimulated immune responses but did not elicit clinical responses in a mixed group of patients with tumors that express carcinoembryonic antigen.¹⁶ Sim-

ilarly, HIV peptide-pulsed allogeneic dendritic cells elicited strong cytolytic T-lymphocyte immune responses but did not affect HIV viral load.¹⁷ These trials all demonstrated that antigen-loaded dendritic cells are effective for stimulating antigen-specific T-cell immune responses. In contrast, Salgaller et al¹⁸ reported that dendritic cells loaded with peptide fragments of prostate membrane-specific antigen stimulated antigen-specific immunity in only two of 82 men with HRP. The low frequency of immune responses to prostate membrane-specific antigen in that study may result from weak immunogenicity of the selected antigen epitopes or poor functionality of the dendritic cells. It has been noted

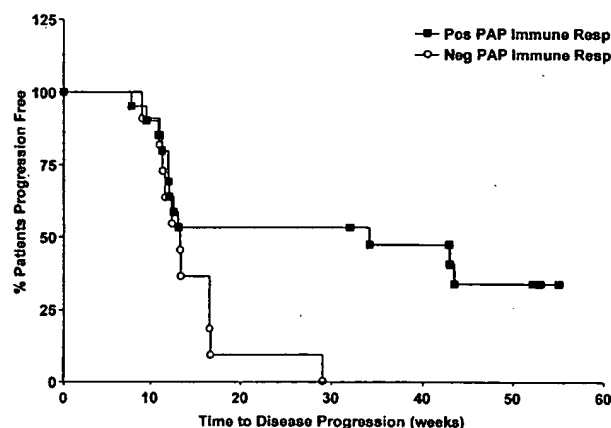


Fig 5. Kaplan-Meier plot of times to disease progression for patients who developed either a T-cell or B-cell response to PAP ($n = 20$) and for patients who did not develop an immune response to PAP ($n = 11$).

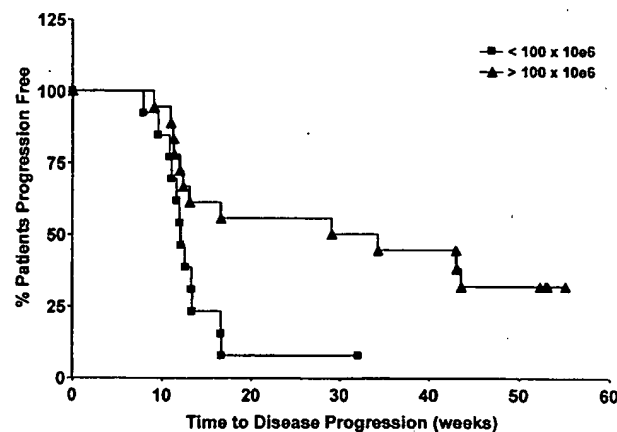


Fig 6. Dose of dendritic cells and time to disease progression. The average dose of dendritic cells infused into each phase I and phase II patient was calculated and compared with the patient's time to disease progression by the Kaplan-Meier method.

previously that dendritic cells pulsed with a whole protein may be more effective than dendritic cells pulsed with HLA class I-restricted peptides for eliciting antigen-specific immune responses in patients with HIV infection.¹⁷ Protein-pulsed dendritic cells may be more effective than single peptide-pulsed dendritic cells for stimulating immunity because of the larger repertoire of antigens present in the protein and the resulting ability to elicit both CD4+ helper cells and CD8+ effector cells.

There was evidence of clinical activity with single-agent Provenge therapy, as revealed by unambiguous PSA declines in some patients. The utility of a decrease of more than 50% in PSA as a marker of response and clinical outcome for men with HRPc remains debated.²⁷⁻²⁹ Nevertheless, a decrease in PSA of more than 50% has been accepted as a reasonable screen for anticancer activity.³⁰ The fact that men with overtly HRPc who had received no other treatment had a sufficient immune response to decrease their PSA levels is provocative.

The observation that time to disease progression correlated with development of an immune response to PAP and to the dose of Provenge is intriguing, but caution is warranted in interpreting these results. The differences in time to disease progression as a function of immune response and cell dose cannot be unambiguously attributed to treatment. It is possible that a lower cell yield or a lower innate immune response to antigens presented by dendritic cells is a function of greater disease burden or increased aggressiveness of disease, so that it would not be surprising that these patients had a shorter time to disease progression. However, many of the patients who received low dendritic-cell doses were part of the phase I dose escalation trial, and the low dose was because of prospectively planned dose levels and not because of an intrinsic defect in the patients' number of dendritic cells. Similarly there were no

apparent differences in the baseline immune function among the different patient groups as assessed by T-cell proliferation responses to the recall antigen influenza. The relationships between clinical benefit and the dose of dendritic cells and the extent of immune response clearly warrant further investigation.

Finally, Provenge seems to be safe and well tolerated. There was no evidence for development of an autoimmune disease caused by cross-reactivity between the PAP antigen and a normal tissue component. This lack of cross-reactivity with normal tissue antigens was predicted from the lack of PAP expression by normal tissues other than the prostate³¹ and from review of gene banks for proteins that express potentially cross-reactive epitopes. An immune response to PAP expressed by normal prostate tissue could result in prostatitis. Although five men had urinary symptoms, none of these were clearly caused by treatment-induced prostatitis. The absence of prostatitis in men with immune responses to PAP is not unexpected as 20 of 31 men had undergone prior local therapy and all men had undergone hormone ablative therapy as well.

In conclusion, active immunotherapy with autologous dendritic cells that were loaded *ex vivo* with a fusion protein containing PAP is a novel approach to prostate cancer immunotherapy. This clinical trial demonstrates that this therapeutic approach is feasible, that treatment is safe and immunologically active, and that clinical activity seems to be present, although proof of clinical benefit will require completion of ongoing controlled randomized trials. This trial establishes the groundwork for future refinements, including optimization of dosing schedule, use in patients with less extensive disease, and possibly in combination with other therapeutic agents or modalities.

REFERENCES

- Greenlee RT, Murray T, Bolden S, et al: Cancer statistics, 2000. *CA Cancer J Clin* 50:7-33, 2000
- Garnick MB: Prostate cancer: Screening, diagnosis, and management. *Ann Int Med* 118:804-818, 1993
- Small EJ, Vogelzang NJ: Second-line hormonal therapy for advanced prostate cancer: A shifting paradigm. *J Clin Oncol* 15:382-388, 1997
- Eisenberger MA, Simon R, O'Dwyer PJ, et al: A reevaluation of nonhormonal cytotoxic chemotherapy in the treatment of prostatic carcinoma. *J Clin Oncol* 3:827-841, 1985
- Dowling AJ, Tannock IF: Systemic treatment for prostate cancer. *Cancer Treat Rev* 24:283-301, 1998
- Banchereau J, Steinman RM: Dendritic cells and the control of immunity. *Nature* 392:245-252, 1998
- Surin MR: Dendritic cells presenting antigen. *Cancer Immunol Immunother* 43:158-164, 1996
- Flamand V, Sornasse T, Thielemans K, et al: Murine dendritic cells pulsed *in vitro* with tumor antigen induced tumor resistance *in vivo*. *Eur J Immunol* 24:605-610, 1999
- Mayordomo J, Zorina T, Storkus J, et al: Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat Med* 1:1297-1302, 1995
- Celluzzi C, Mayordomo J, Storkus W, et al: Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J Exp Med* 183:283-287, 1996
- Ossevoort M, Feltkamp M, van Veen K, et al: Dendritic cells as carriers for a cytotoxic T lymphocyte epitope-based peptide vaccine in protection against a human papillomavirus type 16-induced tumor. *J Immunother Emphasis Tumor Immunol* 18:86-94, 1995
- Hsu FJ, Benike C, Fagnoni F, et al: Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 2:52-58, 1996

13. Lacy MQ, Wettstein P, Gastineau DA, et al: Dendritic cell-based idotype vaccination in post transplant multiple myeloma. *Blood* 94:122a, 1999 (Suppl 10)
14. MacKenzie M, Peshwa MV, Wun T, et al: Efficacy of idotype-pulsed autologous dendritic cells for treatment of advanced refractory multiple myeloma. *Blood* 94:122a, 1999 (Suppl 10)
15. Nestle FO, Alijagic S, Gilliet M, et al: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 4:328-332, 1998
16. Morse MA, Deng Y, Coleman D, et al: A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. *Clin Cancer Res* 5:1331-1338, 1999
17. Kundu SK, Engleman E, Benike C, et al: A pilot clinical trial of HIV antigen-pulsed allogeneic and autologous dendritic cell therapy in HIV-infected patients. *AIDS Res Hum Retroviruses* 14:51-560, 1998
18. Salgaller ML, Lodge PA, Tjoa BA, et al: Monitoring of prostate-specific membrane antigen- (PSMA) specific immune responses and prostate markers in a phase II clinical trial with patients infused with dendritic cells pulsed with PSMA-derived peptides. *Proc Am Assoc Cancer Res* 39:173a, 1998 (abstr 1187)
19. Valone FH, Yang DM, Ruegg CL, et al: Dendritic cell immunotherapy of prostate cancer: Preclinical models of autoimmune prostatitis and early clinical experience. *Cancer Res Therapy Control* (in press)
20. Peshwa MV, Shi JD, Ruegg CL, et al: Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen presenting cells pulsed with prostatic acid phosphatase peptide. *Prostate* 36:129-138, 1998
21. Strang G, Hickling JK, McIndoe G, et al: Human T cell responses to human papillomavirus type 16 L1 and E6 synthetic peptides: Identification of T cell determinants, HLA-DR restriction and virus type specificity. *J Gen Virol* 71:423-431, 1990
22. Schmittl A, Keilholz U, Scheibenbogen C: Evaluation of the interferon- ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *J Immun Meth* 210:167-174, 1997
23. Hornbeck P: *Current Protocols in Immunology* (vol 1). New York, NY, John Wiley and Sons, 1997, pp 2.1.3-2.1.6
24. Abbas AK, KM Murphy, A Sher: Functional diversity of helper T lymphocytes. *Nature* 383:787-793, 1996
25. Burch PA, Kaur JS, Richardson RL, et al: Soluble antigen boost after dendritic cell infusion for immunotherapy of hormone refractory prostate cancer: A phase I trial. *Proc Am Assoc Cancer Res* 40:86a, 1999 (abstr 570)
26. Kugler A, Stuhler G, Walden P, et al: Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. *Nat Med* 6:332-336, 2000
27. Kelly WK, Scher HI, Mazumdar M: Prostate-specific antigen as a measure of disease outcome in metastatic hormone-refractory prostate cancer. *J Clin Oncol* 11:607-615, 1993
28. Sridhara R, Eisenberger M, Sinibaldi V, et al: Evaluation of prostate specific antigen as a surrogate marker for response of hormone refractory prostate cancer to suramin therapy. *J Clin Oncol* 13:2944-2953, 1995
29. Dawson NA: Apples and oranges: Building a consensus for standardized eligibility criteria and end points in prostate cancer clinical trials. *J Clin Oncol* 16:3398-3405, 1998
30. Bubley GJ, Carducci M, Dahut W, et al: Eligibility and response guidelines for phase II clinical trials in androgen-independent prostate cancer: Recommendations from the Prostate-Specific Antigen Working Group. *J Clin Oncol* 17:3461-3467, 1999
31. Solin T, Kontturi M, Pohlmann R, et al: Gene expression and prostate specificity of human prostatic acid phosphatase (PAP): Evaluation by RNA blot analysis. *Biochim Biophys Acta* 1048:72-77, 1990

Adjuvant Immunization of HLA-A2-Positive Melanoma Patients With a Modified gp100 Peptide Induces Peptide-Specific CD8⁺ T-Cell Responses

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Purpose: To measure the CD8⁺ T-cell response to a melanoma peptide vaccine and to compare an every-2-weeks with an every-3-weeks vaccination schedule.

Patients and Methods: Thirty HLA-A2-positive patients with resected stage I to III melanoma were randomly assigned to receive vaccinations every 2 weeks (13 vaccines) or every 3 weeks (nine vaccines) for 6 months. The synthetic, modified gp100 peptide, g209-2M, and a control peptide, HPV16 E7, were mixed in incomplete Freund's adjuvant and injected subcutaneously. Peripheral blood mononuclear cells obtained before and after vaccination by leukapheresis were analyzed using a fluorescence-based HLA/peptide-tetramer binding assay and cytokine flow cytometry.

Results: Vaccination induced an increase in peptide-specific T cells in 28 of 29 patients. The median frequency of CD8⁺ T cells specific for the g209-2M peptide increased markedly from 0.02% before to 0.34% after vaccination

($P < .0001$). Eight patients (28%) exhibited peptide-specific CD8⁺ T-cell frequencies greater than 1%, including two patients with frequencies of 4.96% and 8.86%, respectively. Interferon alfa-2b-treated patients also had significant increases in tetramer-binding cells ($P < .0001$). No difference was observed between the every-2-weeks and the every-3-weeks vaccination schedules ($P = .59$).

Conclusion: Flow cytometric analysis of HLA/peptide-tetramer binding cells was a reliable means of quantifying the CD8⁺ T-cell response to peptide immunization. This assay may be suitable for use in future trials to optimize different vaccination strategies. Concurrent interferon treatment did not inhibit the development of a peptide-specific immune response and vaccination every 2 weeks, and every 3 weeks produced similar results.

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MANY PATIENTS with cutaneous malignant melanoma remain at significant risk of distant recurrence and death following primary surgical excision. For patients at the highest risk of recurrence (eg, > 4 mm primary lesions or node-positive disease) interferon alfa-2b adjuvant therapy has been shown to improve disease-free survival.¹ For patients with melanomas 1 to 4 mm thick, this risk is lower but still significant at 15% to 40%.² These patients have been enrolled in clinical trials to assess the benefit of therapeutic tumor vaccines, which are attractive because of their low toxicity and the responsiveness of melanoma to many immunologically based treatments. A recently reported Southwest Oncology Group study of one such vaccine composed of a lysate of two melanoma cell lines (Melacine; Corixa Corp, Seattle, WA) failed to decrease relapse rates in all

enrolled patients; however, a subgroup of patients who were positive for HLA-A2 and/or HLA-C3 may have benefited from the vaccine.³ Interest in the development of therapeutic vaccines for this group of patients remains high.

Patients in the trial reported herein were vaccinated with the synthetic modified gp100 peptide, gp100:209-217(210M) (hereafter referred to as g209-2M), which was created by altering the anchoring amino acid (methionine in place of threonine) at position 2 of the native gp100:209-217 peptide (hereafter referred to as g209).⁴ This modification enhanced the peptide's affinity for HLA-A2 molecules and increased its immunogenicity in vitro⁴ and in vivo.⁵ Native gp100 is a nonmutated protein differentiation antigen expressed by cells of the melanocytic lineage including melanomas, normal melanocyte, and pigmented retinal cells, but not by other normal tissues. T-cell responses to the native gp100 antigen have been noted in patients with metastatic melanoma who experienced tumor regression following adoptive therapy with tumor-infiltrating lymphocytes (TIL) and interleukin 2 (IL-2).⁶ When the g209-2M peptide was administered with incomplete Freund's adjuvant (IFA) to 11 patients with metastatic melanoma, CD8⁺ T-cell responses to both the native and modified peptides were observed in 10 patients.⁷ Although none of the patients experienced an objective response, three patients exhibited mixed responses with regression of individual lesions. When vaccination was followed by high-dose IL-2 treatment in another group of 31 patients, the response rate was 42%—much higher than the historic response rate of IL-2 alone.⁷ The use of the g209-2M peptide (combined with a tyrosinase peptide) vaccine in the adjuvant setting was studied by Lee et al,⁸ who administered a total of eight vaccines

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over 26 weeks to 48 completely resected stage III or IV melanoma patients. They demonstrated minimal toxicity to the repetitive vaccinations and reported delayed-type hypersensitivity (DTH) skin test responses as well as antigen-specific T-cell responses in nearly all the patients.

The detection of peptide-specific T cells in the peripheral blood has been greatly simplified by the development of the fluorescence-based tetramer-binding assay.⁹ Tetramers are quaternary molecular constructs of four biotin-avidin linked sets of one synthetic HLA heavy chain and a single beta-2 microglobulin light chain folded with noncovalently bound peptide antigens containing eight to 10 amino acids.^{9,10} After conjugation of fluorescent labels, tetramers can be used to specifically label T-cell receptor complexes of defined HLA and peptide specificity. Flow cytometry has been performed using peptide-antigen-specific, HLA class I-restricted, fluorescenciated tetramers to enumerate circulating antigen-specific memory/effector CD8⁺ T cells in human peripheral blood without prior *in vitro* stimulation and expansion of T cells.¹¹⁻¹⁴ Tetramer staining and flow cytometry analysis have been used successfully to quantitate memory/effector T cells specific for cytomegalovirus, human immunodeficiency virus (HIV), and other viral peptides¹⁵⁻¹⁷ and for the detection of CD8⁺ T cells specific for tumor-associated peptides in cancer patients.¹⁸⁻²⁰ The assay has a reported detection sensitivity of 1 in 10,000 cells or 0.01%.²¹ Because this fluorescence-based tetramer-binding assay had the potential to be more reproducible and quantitative than DTH skin testing, and because Weber⁸ had already shown that the gp209-2M peptide induced a positive skin test in almost all patients, we omitted DTH testing from our study.

This report describes a pilot study in which patients with stage I to III melanoma were vaccinated with two HLA-A2-binding synthetic peptides (a modified-self gp100 and nonself human papilloma virus (HPV) 16E7 peptide). We present the clinical results of this study and a detailed description of the immunological monitoring performed with HLA-A2/peptide tetramers for both immunogens.

PATIENTS AND METHODS

Eligibility Criteria

Patients with cutaneous malignant melanoma ≥ 1 mm thick were eligible for this study if they had no evidence of distant metastatic disease and were HLA-A2-positive. Patients determined to be at high risk for recurrence (lesion > 4 mm in thickness or presence of lymph node metastases) were offered interferon alfa (IFN α) along with their vaccinations. Patients had to have a Karnofsky performance status of ≥ 80 , good organ function, and no requirement for treatment with systemic corticosteroids.

Pretreatment Testing

Before enrollment, patients had the following blood tests: complete blood count, complete metabolic panel including lactate dehydrogenase, and HLA-A2 testing. Testing for HIV and screening for hepatitis B and C were performed before leukapheresis. Peripheral blood mononuclear cells were collected at 1 mL/min and less than 3% colorgram during a 3-hour leukapheresis performed before vaccine therapy by the Red Cross to obtain sufficient peripheral blood mononuclear cells for immunologic assays. Imaging studies were done if distant metastatic disease was suspected, but they were not required. In addition to the standard history and physical examination, a baseline ophthalmologic exam was performed.

Treatment

Patients were vaccinated with subcutaneous injections of gp209-2M peptide and a control peptide HPV16E7:12-20. The gp209-2M peptide (National Security Code no. 683472) is an HLA-A2-restricted nine-amino acid epitope derived from melanoma antigen gp100 and has the amino acid sequence IMDQVPFSV. The peptide, which was supplied in vials containing 1 mL of a sterile 1 mg/mL solution for injection with 0.1 N HCL added to adjust the pH, was provided by the Cancer Therapy Evaluation Program (CTEP) under a National Cancer Institute (NCI) investigational new drug (IND) (BB 6123). HPV16E7:12-20 peptide (NSC no. 673925) is an HLA-A2-restricted nine-amino acid peptide of the HPV16E7 protein with the amino acid sequence MLDLQPETT. It was supplied as a 1 mg/mL concentration in vials containing 1 mL of the solution. The peptide was dissolved in sterile water for injection (United States Pharmacopeia). It was supplied by CTEP under a National Cancer Institute IND (BB 6349). Montanide ISA 51 (NSC no. 675756) is an oil-based adjuvant product similar to IFA, which, when mixed with a water-based solution, forms a water-in-oil emulsion. The product is manufactured by Seppic, Inc (Fairfield, NJ), and was provided by CTEP, NCI.

Each vaccine was prepared from frozen vials of the gp209-2M peptide that were thawed at room temperature. Once thawed, 1.2 mL of Montanide ISA 51 was added to the 1.2 mL of peptide solution and emulsified by vortexing for 12 minutes. The HPV16E7 peptide was prepared the same way except that it was initially refrigerated at 4°C and did not need to be thawed.

After the solution was prepared, 1 mL (containing 0.5 mg peptide) was withdrawn from the gp209-2M peptide/adjuvant emulsion and 1 mL (containing 0.5 mg peptide) was withdrawn from the HPV16E7 peptide/adjuvant emulsion and administered into the subcutaneous tissue of two separate sites near each other on one extremity. Another two 1-mL injections of the peptide emulsions were administered in the subcutaneous tissue of two separate sites near each other on a different extremity. Therefore, each time a patient was vaccinated, he or she received a total of 1 mg of each peptide and a total of 2 mL of Montanide ISA 51. In patients who were vaccinated before their sentinel lymph node (SLN) biopsy, one of the sites for the first two immunizations was the site of the primary melanoma. After SLN biopsy, vaccinations were rotated among all extremities except the limb that had undergone lymph node surgery. The abdomen and the upper buttock region were also used as injection sites. Patients were observed in the clinic for a minimum of 2 hours after the initial immunization and for 15 minutes after each subsequent vaccination.

Patients were randomly assigned to two different vaccination schedules: group A received vaccinations every 2 weeks for 6 months (13 total injections), and group B received vaccinations every 3 weeks for 6 months (nine total vaccinations). In patients in whom it had not already been performed, SLN biopsy was done approximately 10 days after the second vaccination in both groups. A wide local excision was done at the same time if one had not previously been performed. Patients whose lymph nodes contained metastatic melanoma also underwent a completion lymphadenectomy. Patients with positive lymph nodes or primary lesions more than 4 mm thick were permitted to receive adjuvant high-dose IFN α at the discretion of the treating physician at the standard dose and schedule according to the package insert.

All patients underwent a leukapheresis similar to the pretreatment leukapheresis 2 to 4 weeks after the completion of vaccine therapy. Patients were seen every 3 months for the remainder of the first year, every 4 months in the second year, and every 6 months thereafter. This protocol was reviewed by CTEP, NCI, and approved by the Providence Health System institutional review board. All patients voluntarily gave their written informed consent before they were screened for eligibility.

Tetramer-Binding Assay

The tetramer-binding assessment of CD8⁺ T cells was performed using four-color flow cytometry analysis. Cell surface staining was carried out using fluorescein isothiocyanate (FITC) anti-CD56, peridinin chlorophyll protein (PerCP)-conjugated anti-CD3, allophycocyanin (APC)-conjugated anti-CD8, phycoerythrin (PE)-labeled gp100 (209-2M), and HPV 16E7:12 to 20 peptide or HIV (polymerase [pol]) HLA-A2-restricted tetramer reagents. The gp209-2M tetramer reagent and a correlated HIV (pol)-negative control tetramer were provided by Dr. Pedro Romero (Ludwig.

Institute-Lausanne, Switzerland); the HPV tetramer reagent and its associated HIV (pol) control tetramer were purchased from Beckman-Coulter Immunomics (San Diego, CA). Tetramer staining analysis data were collected on 5×10^4 to 1×10^5 gated CD8⁺/CD3⁺ T lymphocytes and are expressed in the data tables and figures as the percentage of total CD8⁺ T cells that are positive for tetramer fluorescence for each patient. Tetramer analysis was performed on cryopreserved peripheral-blood mononuclear cells (PBMCs) from the leukapheresis collections before and after vaccination. All tetramer data are expressed as the net specific HPV or gp209-2M tetramer staining after the nonspecific HIV (pol)-mediated tetramer staining is subtracted from each sample.

Cytokine (IFN γ) Flow Cytometry Assay

Cryopreserved PBMCs from vaccinated patients were stimulated directly ex vivo with recall gp209-2M peptide antigen or the native gp209 peptide to determine the number of functionally responsive CD8⁺ T cells as measured by the expression of intracellular IFN γ . Briefly, cells were thawed and washed twice with cold medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum). PBMCs were plated at 1×10^6 cells in 170 μ L of medium per well in a 96-well V-bottom tissue culture plate and allowed to rest overnight at 37°C in a humidified CO₂ incubator (5% CO₂). gp209-2M peptide or the native gp209 peptide at 2 μ g/well and brefeldin A at 2 μ g/well (10 μ g/mL) were added together in a 30- μ L volume to give a final well volume of 200 μ L. Cells were incubated (37°C and 5% CO₂) for an additional 5 hours. Before collection and analysis, EDTA was added to each well (20 μ L of 20 mmol/L EDTA) and incubated at room temperature for 10 minutes to promote recovery of adherent T cells. Cells were centrifuged in the 96-well plate for 5 minutes at 400 \times g and supernatants were aspirated. Cells were then resuspended in 200 μ L/well of 1 \times BD FACS permeabilization buffer (BD Biosciences, San Jose, CA) and incubated for 10 minutes at room temperature. Cells were washed twice (200 μ L fluorescence-activated cell sorting [FACS] wash buffer/centrifugation at 500 \times g for 5 minutes) and resuspended in 200 μ L of FACS wash buffer before monoclonal antibody staining. Cells were stained in the dark at room temperature for 30 minutes using a cocktail of fluorochrome-conjugated mouse antihuman antibodies for IFN γ -FITC (25723.11, immunoglobulin G [IgG_{2b}]), CD69-PE (L78, IgG₁), CD8-PerCp-Cy5.5 (SK1, IgG₁), and CD3-APC (SK7, IgG₁). A separate cocktail of isotype-matched controls IgG_{2a}-FITC (X39), IgG₁-PE (X40), IgG₁-CD8-PerCp-Cy5.5 (SK1), and IgG₁-CD3-APC (SK7) were run in parallel with every test sample. Stained cells were washed in flow wash buffer and fixed in 1% paraformaldehyde for 1 hour at 4°C before acquisition on the same day. Cell acquisition and analysis were performed on the FACSCalibur instrument using CellQuest Pro software (BD Biosciences). Acquisition was performed in list mode by gating on 20,000 CD8⁺-positive T cells within the typical viable lymphocyte region assigned using the forward and side scatter plot.

Statistical Methods

Pre- and postimmunization T-cell immunity to gp209-2M peptide, to HPV16E7 peptide, and to a negative control HLA-A2 HIV peptide (pol) were assessed using HLA-A2/peptide tetramer-specific binding analysis. Within-subject analyses were performed to determine differences between pre- and postimmunization responses to the gp209-2M and HPV peptides and to the negative control HIV peptide after completion of 6 months of vaccination. Because of heterogeneity in the frequency of postimmunization peptide-specific T cells, we report probabilities obtained from nonparametric Wilcoxon signed rank tests for within-subject pre- versus postimmunization responses. The study was designed with a planned comparison of the responses of group A (vaccination every 2 weeks) to the responses of group B (vaccination every 3 weeks). Pre- versus postimmunization response differences were used as criterion measures in between-group (among subjects) analyses. Because of heterogeneity in difference scores, we report probabilities from Wilcoxon rank sum tests for between-group differences. Several unplanned exploratory comparisons were also made; for example, the responses of patients also receiving interferon versus those not receiving interferon, and the responses of node-positive patients versus node-negative patients, elderly patients versus younger patients, and men versus women. As a general strategy to control for possible compounding type I errors in hypothesis tests, we report probabilities less than .01 as significant.

RESULTS

Demographics

Thirty eligible patients were enrolled on this study beginning in May 1999 and ending in May 2001. All patients were evaluable for toxicity and recurrence. There were 17 men and 13 women with a median age of 51 years and a median Karnofsky performance status of 100. Eighteen of the 30 patients (60%) had stage III (node-positive) disease. The patient characteristics are summarized in Table 1 and were similar in both groups A and B. The main differences were the younger median age and the predominance of men in group A.

Toxicities

Toxicity related to the vaccinations consisted mainly of moderate erythema and induration at the sites of vaccination. The induration, which was painful for a few days in some patients; persisted for months after the vaccinations but slowly diminished over time. The persistent induration at the vaccination sites in the upper buttocks and posterior upper arms was bothersome enough to three patients that they refused to continue the vaccinations. Three patients developed ulceration (two patients after their last vaccine) at one or two of the vaccination sites that was accompanied by sterile drainage and required several weeks to heal in some cases. In patients who received IFN α , the toxicities encountered were those that have been described previously.¹ One patient developed a diffuse erythematous, papular rash that resembled a toxic drug eruption during vaccination and IFN α treatment. A biopsy of the skin rash showed a perivascular lymphocytic infiltration of the dermis without evidence of vasculitis, consistent with a drug eruption. Immunofluorescence studies revealed no evidence of deposition of IgG, IgA, or IgM antibodies; C3; or fibrinogen. Both interferon and vaccination were discontinued and then reintroduced. The patient had a milder skin reaction when the interferon was restarted, and because he thought the vaccine injection made it worse, the vaccines were discontinued. No flu-like symptoms were observed in patients receiving the vaccine alone. No retinal abnormalities were observed on ophthalmoscopic examination in any patient, and no autoimmune effects were noted. One patient

Table 1. Patient Characteristics

	Group A (every 2 weeks)	Group B (every 3 weeks)
No. of patients	15	15
Age, years		
Median	48	56
Range	23-80	43-77
Male/female	10/5	7/8
Median KPS	100	100
Lymph node-positive patients	9	9
Lymph node-negative patients	6	6
SLND after vaccine	7	6
IFN during vaccines	7	7
Stopped early	5	3
Progressed during treatment	1	1
Progressed after treatment	3	2

Abbreviations: KPS, Karnofsky performance status; SLND, sentinel lymph node dissection; IFN, interferon.

who had preexisting patchy vitiligo experienced an increase in vitiligo after the vaccinations. One patient developed vitiligo 6 months after vaccination. In total, eight patients stopped treatment early because of progression of disease (two patients), ulceration at the vaccine site (one patient), allergic rash possibly related to the vaccine (one patient), and patient refusal (four patients: three patients because of vaccine intolerance and one patient because of depression related to concomitant IFN therapy).

Clinical Outcome

As of August 1, 2002, the median time on study of the entire patient population is 23 months. Seven patients (all originally stage III) have suffered recurrence of their disease; four patients from group A and three patients from group B. Four patients have died from progressive melanoma (two patients in each group). Three patients are alive with recurrent disease. One patient had an isolated lung nodule resected, which stained positive for gp100, and later developed liver metastases. He is currently receiving biochemotherapy. Another patient had an isolated liver metastasis treated with radiofrequency ablation and later developed brain metastases. The third patient recurred in a regional lymph node basin that was treated with radiation and surgery. The other 23 patients (77%) remain free of recurrent disease.

Immunologic Responses

All but one patient underwent pretreatment leukapheresis, and 23 patients underwent posttreatment leukapheresis. PBMC obtained from peripheral blood draws or leukaphereses performed before vaccination and 2 to 4 weeks after the final vaccine were analyzed for the presence of CD8⁺ T cells capable of binding the g209–2M and HPV16E7 peptide HLA-A2/tetramers. The cryopreserved cells of one patient in group A were not viable after repeated attempts at thawing; therefore, this patient was excluded from the immunologic analysis. Figure 1A depicts the tetramer staining of PBMCs from patient EA8, who exhibited the best response. Neither the pre- nor postvaccination CD8⁺ T cells from this patient exhibited increased binding to the control HIV (pol) tetramers. After nine vaccinations, 8.86% of this patient's circulating CD8⁺ T cells bound g209–2M peptide tetramers. EA8 also mounted a significant response to HPV, with 2.06% of the CD8⁺ T cells binding the HPV tetramer. Two sets of HIV (pol) controls were included because the tetramers for g209–2M and HPV16E7 were obtained from different sources and therefore might be expected to exhibit different levels of background staining. Figure 1B depicts the results from patient EA10, who exhibited a less pronounced response. Again, there was little binding to the control HIV (pol) peptide either before or after vaccination, but there was a significant expansion of g209–2M- and HPV-specific CD8⁺ T cells after vaccination. Note that even though the frequency of g209–2M tetramer-positive CD8⁺ T cells was low (0.78%) after vaccination, the tetramer (PE)-specific staining was bright and well resolved in this histogram depicting events from 50,000 gated CD8⁺ T cells, and the frequency is significantly greater than the frequency of tetramer-binding cells present before vaccination. This level of discrimination was typical of the responses seen in other patients and permitted the detection of responses at levels of 1 tetramer-positive cell/1,000 CD8⁺ T cells.

Overall, 28 of 29 patients demonstrated an increase in g209–2M-specific CD8⁺ T cells after vaccination. Only patient, EA3, failed to respond, and he received only two vaccines before he developed metastatic disease. The median pretreatment frequency of CD8⁺ T cells reactive to the negative control HIV (pol) peptide measured by tetramer analysis of the entire patient population was 2 tetramer-positive cells/10,000 cells CD8⁺ T, or 0.02% (range, 0% to 0.22%). After 6 months of vaccination, this frequency was unchanged at 0.02% (range 0% to 0.12%). The median pretreatment frequency of CD8⁺ T cells to the g209–2M peptide was identical to the control peptide, 0.02% (range, 0% to 0.21%), indicating that before vaccination, most patients had failed to produce an endogenous immune response to this peptide that was detectable by tetramer staining. However, the frequency of peptide-specific CD8⁺ T cells after vaccination increased to a median value of 0.34% (range, 0.03% to 8.86%), an increase that was highly significant ($P < .0001$). After vaccination, eight of 29 patients (28%) exhibited CD8⁺ g209–2M peptide-specific T-cell frequencies greater than 1%, including two patients with exceptionally high frequencies of 4.96% and 8.86%, respectively. A summary of the immune responses induced in all patients is presented in Fig 2. A description of the responses of the patients who stopped treatment early is presented in Table 2. To determine whether the schedule affected the immune response to gp100, tetramer-binding results from patients vaccinated every 2 (group A) or every 3 (group B) weeks for 6 months were compared. Figure 2A displays the CD8⁺ T-cell responses before and after vaccination for the 14 patients in group A. Increases were seen in every patient after vaccination. This result is highly significant ($P < .0001$). The bottom panel of Fig 2 shows similar responses for 15 patients in group B. In this group, 14 of 15 patients showed significant increases in circulating peptide-specific cells ($P < .0001$). No significant difference was observed in the postvaccination increases in g209–2M tetramer positive CD8⁺ T cell responses between patients in groups A or B ($P = .59$). Our results indicate that vaccination schedules every 2 or every 3 weeks were equally effective.

More than half the patients on study had node-positive disease, and most of these patients received concurrent IFN α therapy. To determine whether IFN α had an effect on the gp100-specific response, we compared responses in patients who received IFN α with those who did not. Figure 3A shows CD8⁺ T-cell responses before and after vaccination for 16 patients who did not receive IFN α concurrently with their vaccinations. Postvaccination increases were highly significant ($P < .0001$). Figure 3B shows tetramer-binding responses for 13 patients who received IFN α concurrently with their vaccinations. Postvaccination increases in this group were highly significant as well ($P < .0001$). We performed a hypothesis test to determine whether differences in posttreatment vaccination were different between the group of patients that received interferon and the group that did not. Because patients were not randomly assigned to these groups, a significant difference would be suspect because of possible confounding sources. However, no significant difference was observed ($P = .20$). Hence, peptide vaccination in this study was equally effective among patients who received IFN α and those who did not. These data indicate that

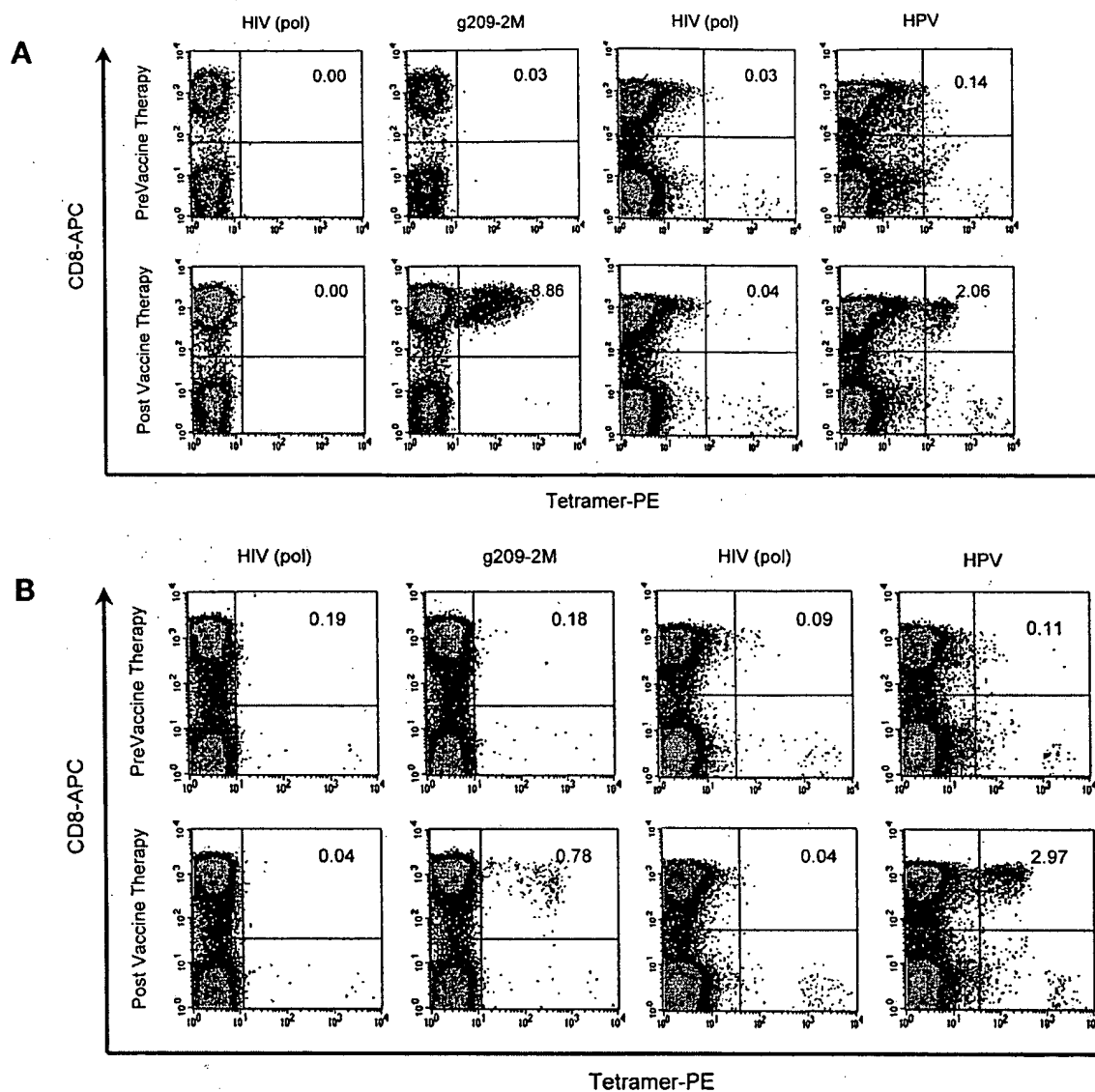


Fig 1. Tetramer-binding of pre- and postvaccination unstimulated peripheral-blood mononuclear cells from patients (A) EA8 and (B) EA10. The ordinate depicts CD8 and the abscissa depicts binding of the indicated peptide tetramers. Human immunodeficiency virus control appears twice because g209-2M and human papilloma virus tetramers were obtained from two different sources (see Methods).

concurrent IFN α treatment does not inhibit the development of a peptide-specific immune response.

We performed additional a posteriori tests to determine whether differences in postvaccination CD8⁺ T increases might be observed between nonexperimental groupings formed after the fact. Because there was no difference between vaccination schedule groups, we collapsed males and females, respectively, across groups A and B and tested for a possible difference caused by sex. No significant difference was observed between male and female postvaccination increases in the CD8⁺ T cell response ($P = .63$). We divided patients into two age groups: younger than 60 years ($n = 22$) and older than 60 years ($n = 17$). The median response for the groups younger than 60 years, 0.64%, was significantly higher than the median response for the group older than 60 years, 0.08% ($P = .0055$). Although the number of patients older than 60 years is small, the results indicate an increase in response for younger patients. We also compared node-positive patients with node-negative patients for

postvaccination increases and found no significant difference between these groups ($P = .18$).

In addition to the modified self-gp100 peptide, all patients were vaccinated with an A2-binding foreign peptide from HPV. Figure 4 presents a comparison of the tetramer staining for the foreign HPV16E7 peptide along with the g209-2M peptide postvaccination for 22 patients from groups A and B who were tested for responses to both peptides. All 22 patients responded to the g209-2M peptide and 20 of 22 patients made an immune response to the HPV peptide vaccine after immunization ($P < .0001$). Patients EA19 and EA30 responded to the gp100 epitope but not to HPV. The single patient who failed to respond to g209-2M was not tested for HPV response because of insufficient cells. Overall, there was no difference between the median responses to the g209-2M peptide and HPV peptides ($P = .92$). However, the response to the g209-2M peptide and the HPV peptide within an individual was not always the same; nine patients made a stronger immune response to the g209-2M

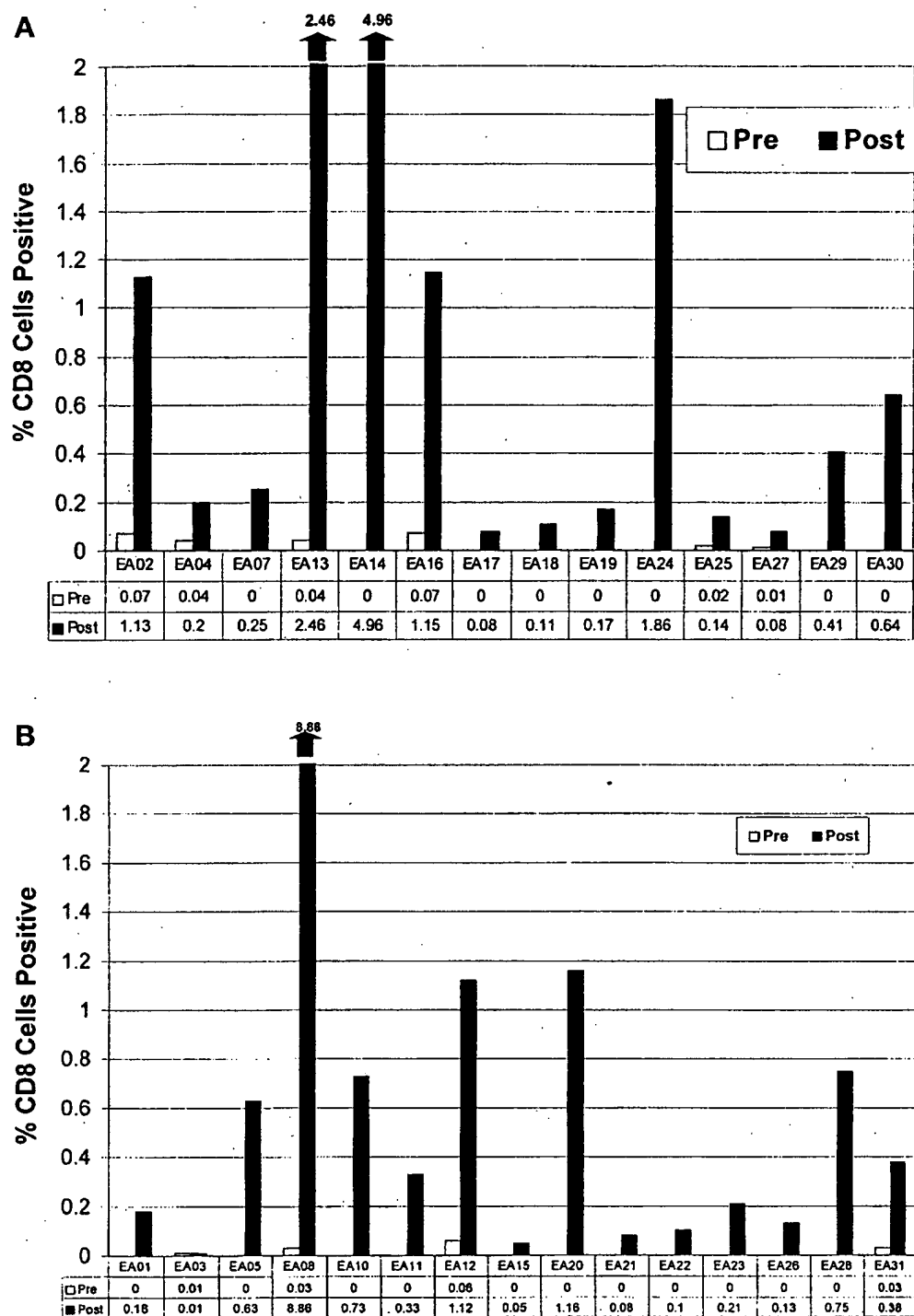


Fig 2. The net percentage of CD8⁺ T cells positive for the g209-2M peptide-tetramer before and after vaccination for patients from (A) group A (2-week schedule) and (B) group B (3-week schedule). The net percentage is the percentage of g209-2M peptide-tetramers minus the percentage of human immunodeficiency virus (polymerase) peptide-tetramers.

peptide than to the HPV peptide, three patients made essentially the same response, and 10 patients made a stronger response to the HPV peptide. If one focuses on the group of 11 patients that had a g209-2M peptide tetramer response of less than 0.5%, five patients had a greater response to HPV, four patients had an equal response, and two patients had a greater response to g209-2M peptide.

The tetramer assay demonstrated that vaccination with the g209-2M peptide led to significant expansion of peptide-specific T cells in almost all patients. However, when performed alone, tetramer binding may not assess the true functional status of the peptide-specific cells.²² Therefore, the functional state of the gp100-specific T cells was determined by direct ex vivo cytokine flow cytometry after gp100 (g209-2M) stimulation to detect

Table 2. Patients Whose Vaccinations Were Stopped Early

Patient	Reason	Group	No. of Vaccines	IFN	% gp100 Tetramer Before Vaccination	% gp100 Tetramer After Vaccination
EA6	Progression	A	5	—	NA*	NA*
EA3	Progression	B	2	+	0.01	0.01
EA16	Ulceration	A	12	+	0.07	1.15
EA24	Rash	A	6	+	0	1.86
EA4	Refusal	A	4	—	0.04	0.20
EA5	Refusal	B	6	—	0	0.63
EA14	Refusal	A	10	+	0	4.96
EA26	Refusal	B	6	+	0	0.13

Abbreviation: IFN, interferon.

*Not available; cryopreserved cells were not viable after thawing.

intracellular production of IFN γ . Previously, it has not always been possible to successfully perform direct ex vivo cytoplasmic recall antigen-stimulated cytokine flow cytometry analysis on freshly thawed cells. Commonly, a period of cognate antigen-stimulated in vitro culture is required before functional analysis. Because the number and function of peptide-specific T cells can be altered by antigen-stimulated in vitro culture, this method may detect immune responses that are not an accurate reflection of the circulating in vivo effector/memory T-cell response. PBMCs from four patients were thawed, allowed to rest overnight, and exposed to the g209–2M peptide and brefeldin A for 5 hours before they were stained to detect intracellular IFN γ (Fig 5). Circulating peptide-specific CD8 $^{+}$ T cells from all four patients made IFN γ in response to in vitro stimulation with the modified g209–2M peptide. In three of four patients there was good correlation between the percentage of g209–2M tetramer-peptide-positive cells and the percentage of IFN γ -positive cells, indicating that most of the cells induced by vaccination were functional and responded to antigen by making IFN γ . In one patient (EA8), only about 50% of the gp100-specific T cells produced IFN γ . These potential differences will be explored further in other patients.

To ensure that the immune response generated to the synthetically altered peptide included T cells that recognized the native peptide, PBMCs from nine patients were stimulated with the native g209 peptide as well as the g209–2M peptide in the direct ex vivo cytokine flow cytometry assay. All patients had detectable numbers of circulating T cells that produced IFN γ in response to the native and the modified g209 peptide; however, the number of T cells that responded to the native peptide was lower than the number that responded to the g209–2M peptide (Fig 6). The response ranged from 52% to 87% of the response to the modified peptide (average 72%).

DISCUSSION

This is one of the first clinical trials in which repetitive peptide vaccination was performed in the adjuvant setting in patients with malignant melanoma. We have shown that nearly all (28 of 29) patients exhibited a significant increase in circulating peptide-specific CD8 $^{+}$ T cells. The immune responses after vaccination appeared to be equivalent whether vaccines were administered every 2 or every 3 weeks; however, the small number of patients in each study arm would exclude only large differences between the two arms. Concomitant IFN α therapy did not hinder

the development of a peptide-specific T-cell response. A post hoc exploratory analysis of age and sex, both of which have been reported to affect immune responses and survival of patients with early stage melanoma,²³ revealed that age did affect the immune response to the gp100 peptide. Patients older than 60 years had a significantly lower peptide-specific response than did younger patients ($P = .0055$). Although it was performed post hoc, this analysis indicates that it would be important to stratify for age in future vaccine trials.

One concern about immunization with a synthetically modified peptide was that the g209–2M peptide-specific T cells might not respond to the native g209 peptide. The nine patients we tested for intracellular IFN γ production by cytokine flow cytometry (CFC) responded to both the native and modified peptide, albeit to a lesser degree with the native peptide. This result parallels the findings of Marincola,²⁴ who demonstrated by tetramer binding analysis the postimmunization induction of T cells specific for the native g209 peptide as well as the modified g209–2M peptide. We did not have a native g209 peptide tetramer reagent available to run in parallel with the g209–2M peptide tetramer, so we were unable to determine the differences in g209 and g209–2M tetramer binding cells. In addition, we have been able to show that the tetramer-binding CD8 $^{+}$ T cells induced by peptide vaccination produced IFN γ in response to stimulation with A2-positive/gp100-positive melanoma cell lines, demonstrating that T cells produced in response to vaccination with the modified peptide recognized naturally processed antigen on the surface of tumor cells.²⁵

In previous studies, when the g209–2M peptide in IFA was used to immunize patients with metastatic melanoma, the majority responded to both the native and the modified g209–2M peptide.⁷ However, immune responses were not observed in freshly isolated uncultured lymphocytes; they were detected only after restimulation with the immunizing peptide in vitro for at least 4 days. The in vitro stimulation and expansion of T cells with antigenic peptides and cytokines can alter the frequency and functional characteristics of these cells. Therefore, immune function and phenotype assays using in vitro stimulation-expanded T cells may not always reflect the fidelity of the in vivo antitumor immune response. Direct ex vivo measurement with peptide-HLA tetramer complexes provides a more accurate representation of the true frequency of circulating peptide-specific T cells in vivo.

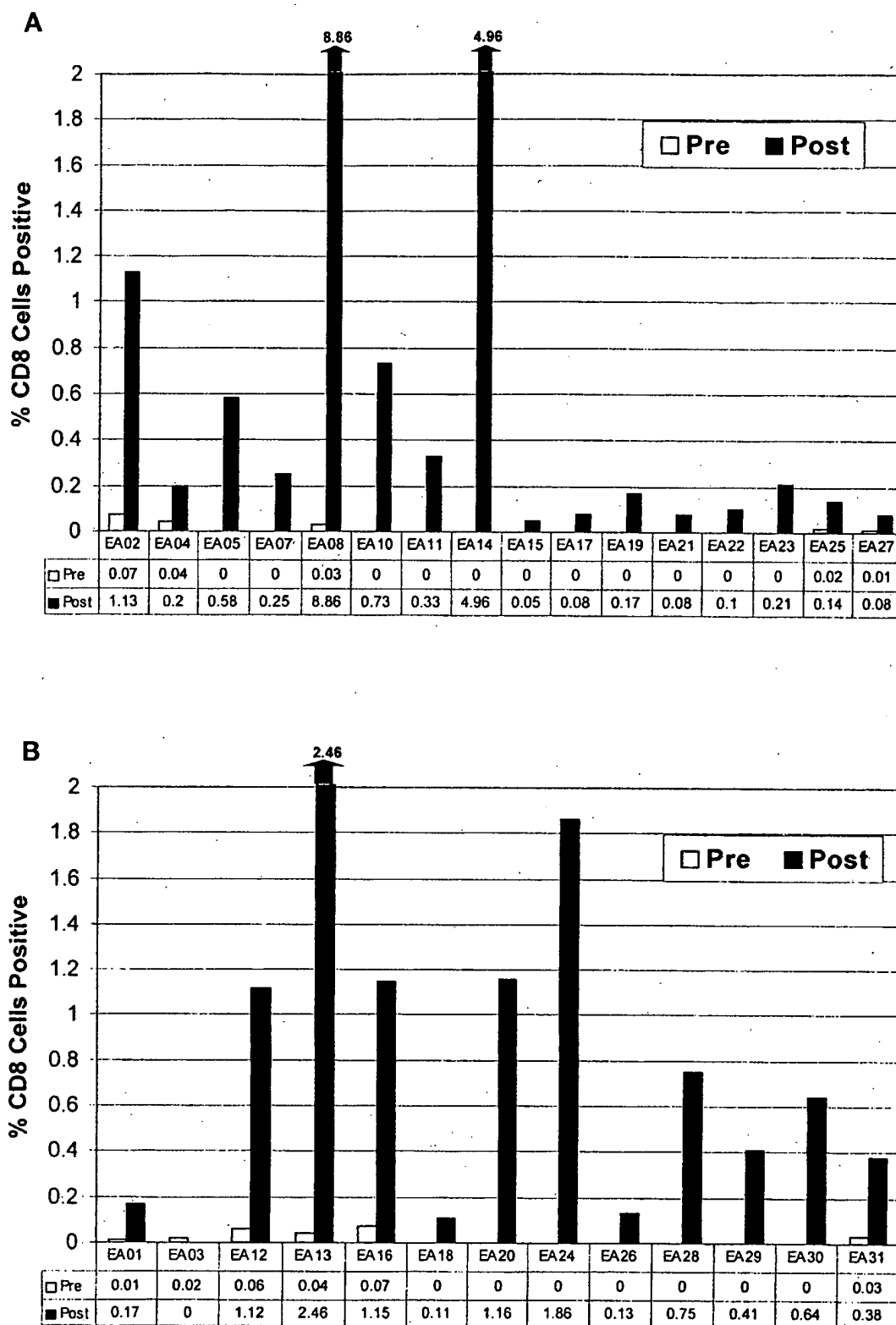


Fig 3. The net percentage of CD8⁺ T cells positive for the g209-2M peptide-tetramer before and after vaccination for patients (A) who did not and (B) who did receive concurrent adjuvant interferon alpha. The net percentage is the percentage of g209-2M peptide-tetramers minus the percentage of human immunodeficiency virus (polymerase) peptide-tetramers.

Using HLA/g209-2M peptide tetramer analysis, Marincola²⁴ reported that all seven metastatic melanoma patients immunized with g209-2M peptide in IFA alone demonstrated significant increases in peptide-specific T-cell precursor frequencies after

vaccination. The responses ranged from 0.2% to 2.4%, with two of the seven patients showing a response greater than 1%. After vaccination, all seven patients also exhibited an increased frequency of circulating CD8⁺ T cells that recognized the native

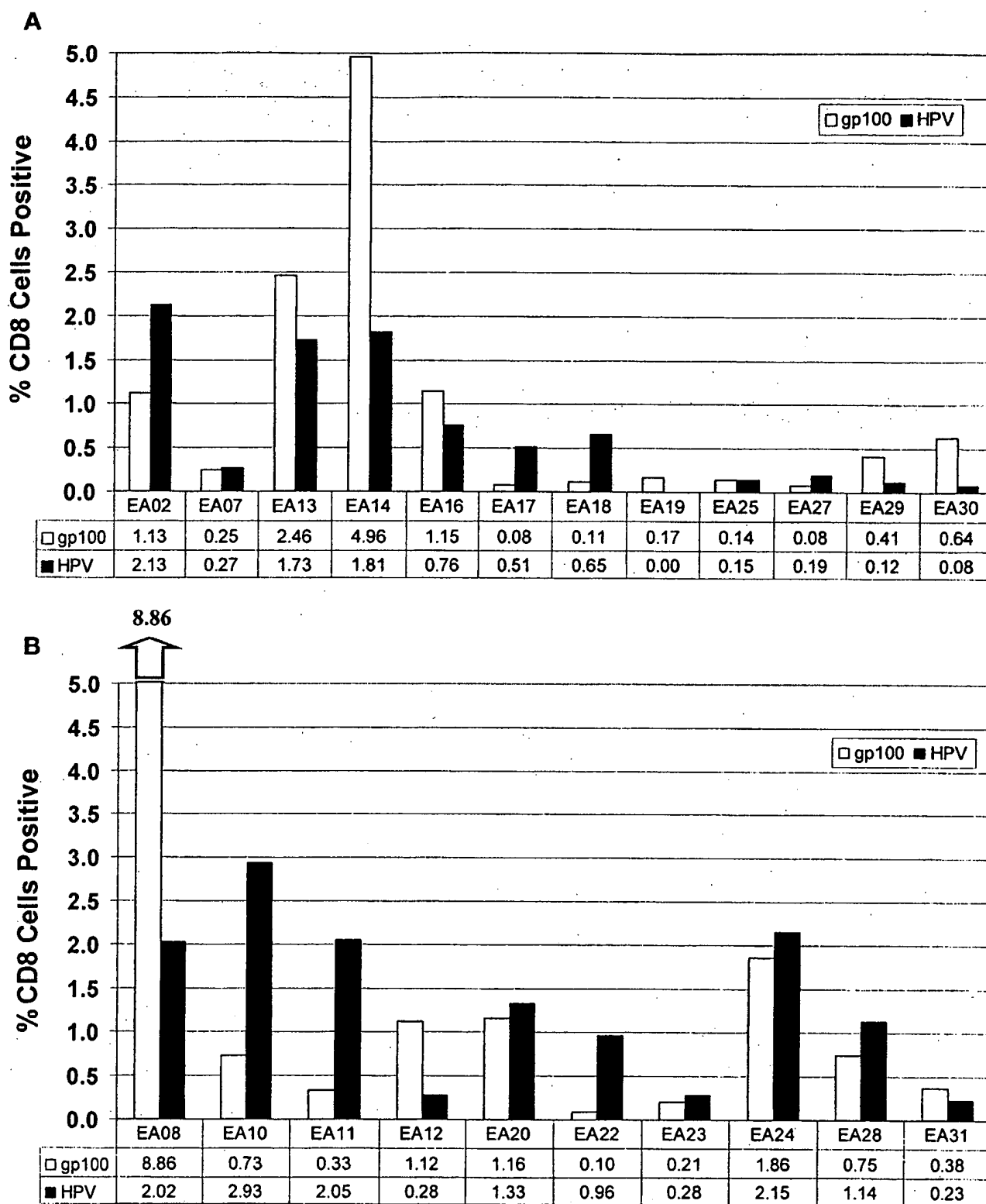


Fig 4. The net percentage of CD8⁺ T cells positive for the g209-2M peptide-tetramer and the HPV peptide-tetramer before and after vaccination for selected patients from (A) group A and (B) group B. The net percentage is the percentage of g209-2M or HPV peptide-tetramers minus the percentage of human immunodeficiency virus tetramers.

g209 peptide, but at a lower frequency than for the modified peptide. In this study, similar increases, which ranged from 0.05% to 8.86%, were observed after vaccination; in eight of 29 patients (28%), more than 1% of the CD8⁺ T cells recognized the g209-2M peptide, including in two patients with the excep-

tionally high frequencies of 4.96% and 8.86%, respectively. Lee et al⁸ reported g209-2M peptide-specific CD8⁺ T-cell frequencies that ranged between 0% and 2.5% (mean, 0.03%) in 37 resected stage III or IV melanoma patients who received eight g209-2M peptide vaccines in IFA during 6 months. The higher

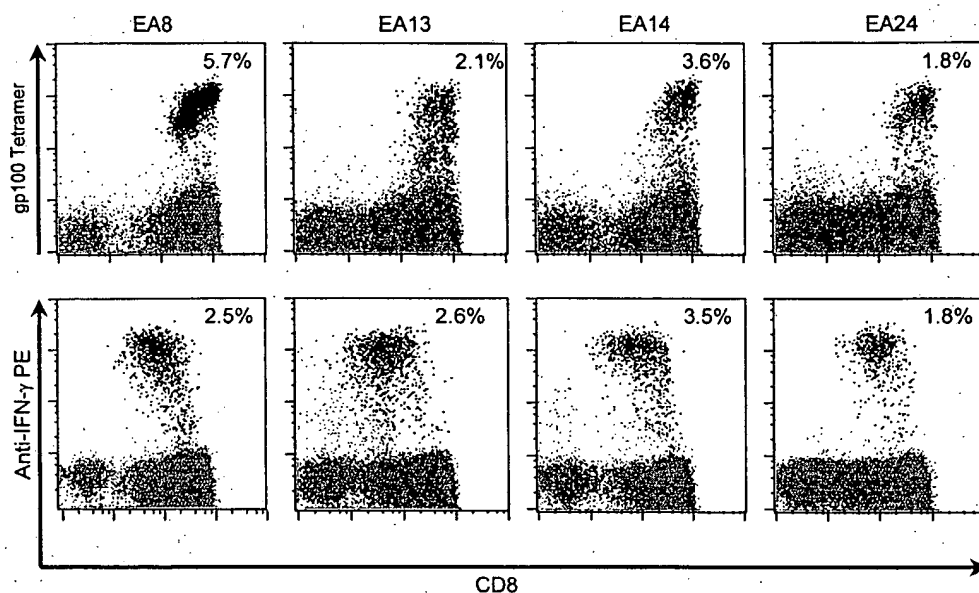


Fig 5. The top panel shows the g209-2M peptide-tetramer and CD8⁺ staining from four patients for postvaccination peripheral-blood mononuclear cells that were also thawed and stimulated *in vitro* for 5 hours with the g209-2M peptide and stained to detect the presence of CD8⁺ and intracellular interferon gamma (bottom panel).

frequency of peptide-specific T cells observed in our study compared with the study by Lee et al⁸ might be caused by the difference in patient population (there were no stage IV patients in our study) or by differences in the technique used in the tetramer assay used to detect the g209-2M peptide-specific CD8⁺ T-cells.

Although there was good correlation between the levels of CFC (IFN-γ) response and tetramer staining for most of the patients studied (Fig 5), not all tetramer-binding cells in every patient will be functional. This is illustrated by the response of

patient EA8, in whom less than 50% of the tetramer-binding cells produced IFN-γ (Fig 5). They may produce other cytokines or could conceivably have become anergic, as reported by Lee et al²⁰ for circulating tetramer-binding CD8 cells in patients with metastatic melanoma. Because HLA-restricted peptide-specific tetramer staining permits the quantitative comparison of different vaccination strategies in different patient groups monitored by different laboratories, it could serve as at least one of the measurements by which an optimal vaccination strategy may be chosen.

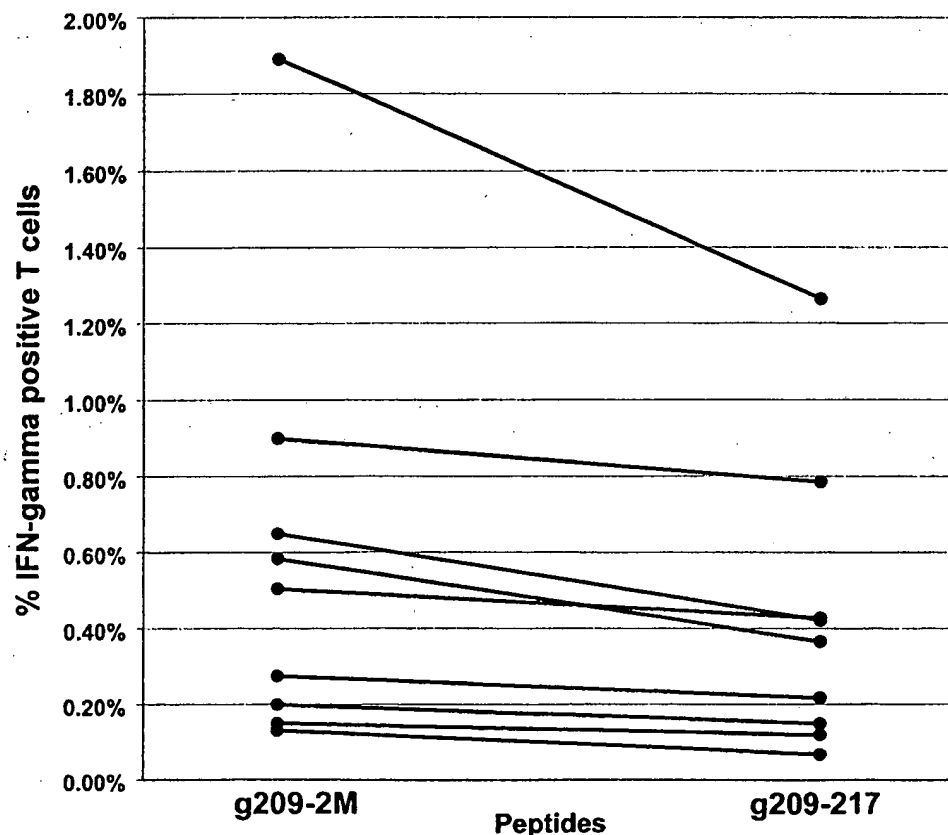


Fig 6. The net percentage of CD8⁺ interferon gamma-positive T cells in response to a 5-hour stimulation *in vitro* with the g209-2M peptide and with the native g209 peptide at 4 μg/well for nine patients after vaccination.

Vaccination with the nonself HPV16E7 peptide was performed as a control to observe the immune response in T cells that would not have been subject to negative selection in the thymus. The fact that 20 of 22 patients (91%) studied responded to this peptide indicates that it could serve as a positive control in trials of other new peptide vaccines.

Previous clinical trials of vaccination with the g209-2M peptide showed that concomitant administration of IL-2, IL-12, or granulocyte-macrophage colony-stimulating factor actually decreased the circulating peptide-specific T-cell precursor frequency.²⁶ We were interested in whether the patients in our study who received IFN α in addition to their vaccines would demonstrate a similar effect. Overall, no significant difference was seen in the two groups, indicating that IFN α did not diminish the effectiveness of the g209-2M peptide in IFA vaccine, a finding that complements the observations of Kirkwood et al,²⁷ who showed that high-dose IFN α did not diminish the antibody response to the GM2 ganglioside. In fact, we observed a trend toward enhancement of the vaccine by IFN α , indicated by the observation that seven of 13 IFN α -treated patients had more than 0.5% tetramer-positive cells versus five of 16 patients who did not receive IFN α . It should be noted that patients were not randomly assigned to receive IFN α and that this population could have been skewed because it generally excluded older, less medically fit patients who might also have had less response to immunization. However, we believe that the data support inclusion of IFN α treatment in clinical trials of other immune strategies in patients with stage III melanoma.

The lack of tumor regression in patients with metastatic melanoma immunized with the g209-2M peptide alone may not have been a result of peripheral tolerance, but it could have been because the overall quantitative immune response was too low. The circulating T-cell response in viral infections or autoimmune disease models measured by epitope/HLA tetramers is much higher. In HIV patients, an inverse correlation has been reported between HIV-specific cytotoxic T lymphocyte frequency and the viral RNA load, and in recent studies,^{15,16} patients whose frequency is approximately 2% remain asymptomatic. Furthermore, animal models indicate that the ability to clear tumor correlates with the intensity of the vaccine-elicited response.²⁸ The target for an immunization strategy in cancer patients is presently unknown, but it seems reasonable to attempt to achieve numbers of circulating tumor-specific memory/effector T cells in the range seen with infections. Despite the fact that our patient population had a low tumor burden and received nine to 13 vaccinations over 6 months, peptide-specific T-cell frequencies more than 1% and more than 2% were achieved in only 28% and 10% of patients, respectively. This indicates that better immunization strategies are necessary. Our study indicates that enumeration of vaccine-elicited T cells by HLA/peptide tetramers is an excellent way to evaluate improvements in vaccine development and immunization strategies. However, for optimal results, a functional assay such as the enzyme-linked immunospot assay or CFC should also be used.²²

REFERENCES

- Kirkwood JM, Strawderman MH, Ernstoff MS, et al: Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: The Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol* 14:7-17, 1996
- Balch CM, Soong SJ, Gershenwald JE, et al: Prognostic factors analysis of 17,600 melanoma patients: Validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 19:3622-3634, 2001
- Sosman JA, Unger JM, Liu P-Y, et al: Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: Impact of HLA class I antigen expression on outcome. *J Clin Oncol* 20:2067-2075, 2002
- Parkhurst MR, Salgaller ML, Southwood S, et al: Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J Immunol* 157:2539-2548, 1996
- Salgaller ML, Marincola FM, Cormier JN, et al: Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptides. *Cancer Res* 56:4749-4757, 1996
- Kawakami Y, Eliyahu S, Jennings C, et al: Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 154:3961-3968, 1995
- Rosenberg SA, Yang JC, Schwartzentruber DJ, et al: Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 4:321-327, 1998
- Lee P, Wang F, Kuniyoshi J, et al: Effects of interleukin-12 on the immune response to a multi-peptide vaccine for resected metastatic melanoma. *J Clin Oncol* 19:3836-3847, 2001
- Altman JD, Moss PA, Goulder PJ, et al: Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96, 1996
- Garboczi DN, Hung DT, Wiley DC: HLA-A2-peptide complexes: Refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc Natl Acad Sci U S A* 89:3429-3433, 1992
- Moldrem JJ, Lee PP, Wang C, et al: A PR1-human leukocyte antigen-A2 tetramer can be used to isolate low-frequency cytotoxic T lymphocytes from healthy donors that selectively lyse chronic myelogenous leukemia. *Cancer Res* 59:2675-2681, 1999
- Hoffmann TK, Donnenberg VS, Friebe-Hoffmann U, et al: Competition of peptide-MHC class I tetrameric complexes with anti-CD3 provides evidence for specificity of peptide binding to the TCR complex. *Cytometry* 41:321-328, 2000
- Whelan JA, Dunbar PR, Price DA, et al: Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J Immunol* 163:4342-4348, 1999
- Romero P, Pittet MJ, Valmori D, et al: Immune monitoring in cancer immunotherapy. Ernst Schering Res Found Workshop 30:75-97, 2000
- Ogg GS, Jin X, Bonhoeffer S, et al: Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2103-2106, 1998
- Gray CM, Lawrence J, Schapiro JM, et al: Frequency of class I HLA-restricted anti-HIV CD8⁺ T cells in individuals receiving highly active antiretroviral therapy (HAART). *J Immunol* 162:1780-1788, 1999
- He XS, Rehmann B, Lopez-Labrador FX, et al: Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc Natl Acad Sci U S A* 96:5692-5697, 1999
- Romero P, Dunbar PR, Valmori D, et al: Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med* 188:1641-1650, 1998
- Ranieri E, Kierstead LS, Zarour H, et al: Dendritic cell/peptide cancer vaccines: Clinical responsiveness and epitope spreading. *Immunol Invest* 29:121-125, 2000
- Lee PP, Yee C, Savage PA, et al: Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* 5:677-685, 1999

21. Bieganska K, Hollsberg P, Buckle GJ, et al: Direct analysis of viral-specific CD8⁺ T cells with soluble HLA-A2/Tax11-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J Immunol* 162:1765-1771, 1999
22. Keilholz U, Weber J, Finke JH, et al: Immunologic monitoring of cancer vaccine therapy: Results of a workshop sponsored by the Society for Biological Therapy. *J Immunother* 25:97-138, 2002
23. Schuchter L, Schultz DJ, Synnestvedt M, et al: A prognostic model for predicting 10-year survival in patients with primary melanoma. The Pigmented Lesion Group. *Ann Intern Med* 125:369-375, 1996
24. Lee KH, Wang E, Nielsen MB, et al: Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J Immunol* 163:6292-6300, 1999
25. Meijer SL, Dols A, Hu H-M, et al: gp100 peptide vaccination of HLA-A2+ melanoma patients induces peptide-specific T cells capable of recognizing naturally processed antigen. *Proc Am Assoc Cancer Res* 43:561, 2002 (abstr 2786)
26. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al: Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J Immunol* 163:1690-1695, 1999
27. Kirkwood JM, Ibrahim J, Lawson DH, et al: High-dose interferon alfa-2b does not diminish antibody response to GM2 vaccination in patients with resected melanoma: Results of the Multicenter Eastern Cooperative Oncology Group Phase II Trial E2696. *J Clin Oncol* 19:1430-1436, 2001
28. Perez-Diez A, Spiess PJ, Restifo NP, et al: Intensity of the vaccine-elicited immune response determines tumor clearance. *J Immunol* 168:338-347, 2002